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THE EFFECTS OF DAILY INJECTIONS OF BOVINE ANTERIOR PITUITARY EXTRACT UPON THE DEVELOPING ALBINO RAT

(Two figures)

GEORGE EDWIN JOHNSON AND EVERETT DUANE SAYLES

Contribution No. 109 from the Department of Zoölogy, Kansas Agricultural Experiment Station, Manhattan, Kansas

INTRODUCTION

AN ASSOCIATION between the pituitary gland, or hypophysis, and growth such as occurs in acromegaly and giantism has been recognized for some time. While recent experiments have indicated that the anterior lobe of the pituitary affects growth and sexual development, different methods have not produced identical results. This has pointed to the need for more work and has led to the present study.

Early work included feeding and transplantation of the whole pituitary gland. Feeding usually stimulated growth or development in larval amphibians but produced no striking effects in young mammals. Transplantation has usually been reported as somewhat stimulating to growth (Clairmont and Ehrlich, 1909; Exner, 1910; Shafer, 1909, 1911; Smith, 1926, 1927*b;* and Allen, 1928). Daily intramuscular transplants of fresh anterior pituitary appears to stimulate sexual development in hypophysectomized female rats (Smith, 1926) as well as in normal young rats and mice (Smith and Engle, 1927). Three transplants usually caused mice 17 days old to

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mature in 36 hours instead of at the age of 35 days. Superovulation was produced, as many as forty-eight ova being found in one horn of the uterus of one mouse in contrast to seven, the highest number in one horn among one hundred normal mice. A slight acceleration of sexual development was reported in the males. By securing maximum response to transplants, Engle (1928) produced cystic follicles and cessation of ovulation. Early injections of the anterior pituitary extract appear, from recent work, to have been ineffective largely because the methods of preparation of the extract destroyed the potency of the hormone, or hormones. Isovesco (1913) produced growth in very young rabbits by the injection method. Increased growth, inhibition of oestrus, inhibition of growth of the uterus, but increased size of the ovaries by an excessive development of lutein tissue produced in young rats by daily injections was reported by Evans and Long (1921, 1922). Growth was obtained by Robertson and Ray (1925) in mice; by Evans (1924) and Evans and Simpson (1925, 1926, 1927) in rats; and by Putnam, Teel, and Benedict (1928) in rats and dogs but not in rabbits. Further proof of the presence of a growth hormone in the extract of the anterior lobe of the pituitary was produced by Smith (1926, 1927*b*) in hypophysectomized rats, by Flower and Evans (1925) in thyroidectomized rats, and by van Wagenen (1927, 1928) in castrated rats.

METHODS

Pituitary glands were secured from the Armour Packing Company of Kansas City. They were removed from heads of cattle in the afternoon, chilled overnight, packed in ice and shipped to Manhattan the next forenoon, where they arrived still cold at 2:15 P.M.

The extract of the anterior pituitary glands was prepared partly following Evans' (1924) method, but more nearly according to Dr. T. Putnam's modification kindly sent us by letter previous to publication (Putnam, Teel, and Benedict, 1928).[1] Because of changes from both of these methods, the one used in this work will be described.

[1] Preliminary work on six litters of rats with extract prepared by centrifuging the crushed gland resulted in the death of most of the rats, so this method was abandoned as unsatisfactory where the extract had to be prepared from glands several hours old and used over a period of days. The few rats which survived the early injections of this extract are included in the tables of this paper (Groups II and III).

The anterior lobes of the pituitary glands were removed under aseptic conditions, weighed and rinsed with very dilute alcohol to remove excess blood. They were ground very fine with sterile sand in a mortar. To the resulting mixture was added 1 cc. of 0.2 normal solution of sodium hydroxide for each gram of the anterior lobes used. This was allowed to stand 3–48 hours, when it was emptied into a cloth strainer and allowed to drain for 5–24 hours. The glandular material and sand in the cloth were then placed under pressure to obtain the remaining free fluid. Using litmus paper and phenol red as indicators, the filtrate was then neutralized (to a pH of 7.0–7.2) with a 0.2 normal solution of acetic acid. A fine precipitate of proteins resulted. The fluid was filtered through glass wool and filter paper, or only through coarse filter paper frequently changed as the pores clogged. The fluid, which was clear and light red, was measured and sodium benzoate added to make a 0.5 per cent solution. A new extract was made practically every week. All the processes which required any length of time were carried out in a refrigerator at an average temperature of about 8° C.

Litter-mate rats (albino except two black ones in Group VIII) were weighed at 14 or 15 days of age and divided into two groups with animals of similar weight and sex paired against each other, taking the lighter of each pair for anterior pituitary injection except in one case. From this time the experimental animals were injected intraperitoneally with 0.2 cc. of the extract daily, and the dosage increased gradually to a maximum dose representing 1.5 gm. of the beef anterior pituitary gland by the time most of the animals in the group weighed 100 gm. each. Generally in the early part of the injection work, 1 cc. extract was made from 0.5 gm. or more of gland, whereas in the latter 1 cc. represented 1 gm. or more of gland, so that no animal received more than 1.5 cc. extract at any time. Weights were taken every 5 days. The controls were injected with a 0.9 per cent solution of sodium chloride equal in amount to the fluid used for the experimental animals. At 150 days the rats were killed and autopsied to make certain that no abnormal condition had been produced in the viscera which would have modified growth. After the testes had been measured, they and the uteri and ovaries were removed and fixed in Bouin's fluid. Measurements from the

tip of the nose to the anus and from the anus to the end of the tail (if the tail had not been injured) were made. To get a measurement of a group of long bones, the entire left hind leg was measured from the acetabulum to the tip of the claw of the third toe. Measurements were made three times, and the average recorded.

RESULTS

EFFECTS OF THE EXTRACT ON GROWTH

The weights of the experimental and control females taken at 5-day intervals are given in Table I and of the males in Table II. The bold-faced type indicates the heavier animal of one pair, consisting of an experimental and a control animal. The omission of animals which died much before the end of the experiments from accident or other cause made it necessary in three cases to compare experimental females with controls not quite 1 gm. lighter (VII, 2 vs. 4, 7 vs. 11, and X, 6 vs. 7) and one experimental male with a control 3 gm. lighter (XI, 5 vs. 7). This, however, is balanced in the case of the females and more than balanced in the males by the heavier controls in other pairs. It is also seen (Table I) that the control females equaled or exceeded the weights of the three originally heavier experimental females in three to six of the first nine weighings.

It is to be seen (Table I) that each experimental female became heavier than its litter-mate control at respectively 30, 95, 60, 40, 40, 60, and 45 days of age. The average weight of the experimentals became greater than that of their controls at 45 days of age, showing a moderate but not marked response in young animals during 30 days of injection. Even the three experimental females which were slightly heavier at first failed to gain as rapidly as the controls for a time, but forged ahead again at 95, 60, and 40 days, respectively. The average weight of the experimentals rose definitely above that of the controls after 45 days of age. It should be noted that the average of all the experimental females, including those without controls, is little different from that of the seven experimental females which have controls.

The increase in growth of the experimental females is also seen in Figure 1, 1 vs. 2, 6 vs. 3, and Figure 2, 11 vs. 6. The great tem-

TABLE I

WEIGHTS OF FEMALES IN GRAMS, TAKEN EVERY 5 DAYS FROM THE 15TH TO THE 150TH DAY OF LIFE. HEAVY TYPE INDICATES THE HEAVIER ANIMAL OF EACH PAIR WHICH CONSISTS OF ONE EXPERIMENTAL AND ONE CONTROL RAT OF THE SAME LITTER

GROUP NO.	DAYS																										
	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150
Experimental Females, Each of Which Has a Control Given in the Same Position in the Control List Below																												
III, 7	16.8	23	34	**48**	**63**	**79**	**94**	**100**	**107**	**116**	**127**	**127**	128	128	131	132	128	141	150	**161**	**184**	**207**	**210**	**217**	**225**	**237**	**245**	**251**
VII, 2	**18.1**	**22**	**27**	36	44	45	60	70	81	88	98	103	113	120	134	154	**173**	**180**	**187**	**194**	**211**	**218**	224	237	235	231	237	**238**
VII, 7	**18.5**	**22**	**26**	34	**46**	**61**	**76**	84	98	**108**	**117**	**132**	**141**	**149**	**160**	**177**	**184**	**193**	**202**	**210**	**222**	**227**	**241**	**243**	248	255	257	262
X, 6	**26.1**	35	43	57	67	**86**	**110**	**130**	**146**	**161**	**171**	**185**	**200**	**211**	**225**	**235**	**245**	**247**	**253**	**250**	**259**	**259**	**255**	**264**	**261†**	**259†**	**286**	**278**
XI, 1	25.4	**33**	38	49	58	**70**	**83**	**103**	**120**	**135**	**139**	**145**	**160**	**176**	**179**	**190**	**204**	**214**	**218**	**217**	**222**	**226**	**232**	**238**	**230†**	**221†**	**248**	**247**
XI, 6	26.8	33	38	48	60	70	85	102	**111**	**122**	**129**	**139**	**152**	**173**	**175**	**185**	**195**	**206**	**214**	**215**	**224**	**234**	**242**	**239**	**234†**	**225†**	**255**	**262**
XII, 11	17.9	20	25	36	43	48	**74**	**88**	**104**	**114**	**132**	**147**	**162**	**168**	**177**	....	**195**	**192**	**197**	**207***	**217***	**240***	**277***	**248**	**248**	**263**	**254**	**240**
Average	21.4	27	33	44	54	66	**83**	**97**	**110**	**121**	**130**	**140**	**151**	**161**	**169**	**179**	**189**	**196**	**203**	**208***	**220***	**230***	**240***	**241**	**240†**	**242†**	**255**	**254**
Experimental Females without Controls or Showing Injury by First Injection (X, 5)																												
II, 3	15.6	21	27	39	55	74	89	104	120	127	138	151	154	162	170	173	180	183	**194***	**210***	**235***	**255***	218	231	241	256	273	**301**
IX, 2	23.0	34	43	52	61	76	93	113	118	128	138	155	173	186	213	221	....	238	248	259	268	276	280	282	280	283	289	279
IX, 5	24.1	35	45	52	65	72	93	109	127	139	146	145	152	166	191	199	....	219	228	238	248	257	257	266	277	270	266	268
X, 2	23.0	32	39	57	68	84	106	121	140	153	159	171	196	198	200	213	222	223	226	229	239	241	244	249	250†	241†	256	259
X, 5	17.7	26	32	42	46	60	69	88	105	119	130	147	158	167	169	182	198	202	203	204	210	211	213	214	211†	198†	219	215
XIII, 1	24.9	33	39	53	62	61	....	91	106	118	127	140	145	169	171	188	179	195	193	198	210	218	224	244	253	265	273	287
Average of all females	21.4	28	35	46	57	68	79	100	114	125	135	145	156	167	177	187	200	202	209*	215*	227*	236*	240	244	246†	247†	258	261
Control Females																												
III, 6	**17.2**	**24**	34	44	59	74	85	94	101	108	119	125	**129**	**132**	**137**	**142**	**143**	**142**	**155**	159	173	189	206	187	189	197	191	207
VII, 4	17.2	20	24	**37**	**46**	**60**	**72**	**80**	**92**	**104**	**115**	**127**	**137**	**142**	**150**	**160**	166	169	173	**185***	**195***	**206***	**248***	200	208	213	206	212
VII, 11	17.9	21	25	34	43	57	71	**86**	98	95	102	111	122	129	130	153	154	161	162	167	**181***	**192***	**221***	**200***	dead at full term			
X, 7	25.3	35	**44**	**63**	**71**	80	102	112	130	142	153	163	175	185	187	197	209	213	226	232	237	241	237	250	**254†**	**239†**	254	258
XI, 3	**25.6**	32	**39**	49	58	66	79	89	99	104	112	115	123	134	139	143	151	146	157	153	158	164	167	169	**178†**	**161†**	173	185
XI, 2	**27.0**	**35**	**39**	**50**	**65**	**75**	**92**	**107**	**115**	120	127	135	140	150	153	157	160	162	177	170	176	184	181	180	**164†**	**156†**	177	181
XII, 6	**19.0**	**23**	**30**	**39**	**48**	**60**	72	84	94	102	112	124	140	138	152	....	167	**176***	**185***	**201***	**225***	195	200	203	**204†**	**200†**	192	192
Average	21.3	27	**34**	**45**	**56**	**67**	82	93	104	111	120	129	138	144	151	159	164	**167***	**176***	**181***	**192***	**196***	**209***	**198***	**171†**	**167†**	170	176

* Weight taken during pregnancy, or averages which are affected by pregnancy of one or more individuals.

† Low weight produced by insufficient feeding for several days. Also indicates averages affected.

TABLE II

WEIGHTS OF MALES IN GRAMS, TAKEN EVERY 5 DAYS FROM THE 15TH TO THE 150TH DAY OF LIFE. HEAVY TYPE INDICATES THE HEAVIER ANIMAL OF EACH PAIR WHICH CONSISTS OF ONE EXPERIMENTAL AND ONE CONTROL RAT OF THE SAME LITTER

GROUP NO.	DAYS																											
	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150
Experimental Males, Each of Which Has a Control Given in the Same Position in the Control List Below																												
III, 10	17.1	22	31	41	57	75	84	**96**	**114**	**124**	**134**	138	144	146	153	164	163	**181**	**199**	**207**	**220**	**230**	**232**	**243**	**257**	**267**	**275**	**284**
VIII, 10	18.0	21	26	34	42	61	62	74	85	99	109	108	112	107	130	150	157	169	182	189	200	209	221	227	233	244	247	253
XI, 5	**26.8**	**34**	**43**	**53**	**69**	**79**	**100**	**123**	**128**	**140**	**153**	**163**	**179**	**194**	**205**	**217**	**223**	**236**	**245**	**245**	**260**	**268**	**279**	**268**	**252**	**246**	**279**	**293**
XII, 1	15.4	19	23	34	45	62	74	86	102	117	130	**144**	**152**	**160**	**172**	....	**190**	**202**	**210**	**216**	**222**	**225**	**231**	**239**	**239**	**254**	**263**	**264**
XII, 5	19.3	23	29	44	60	80	**89**	**103**	**116**	**127**	**140**	158	**173**	**182**	**200**	....	**221**	**237**	**243**	**243**	**247**	**264**	**270**	**278**	**283**	**289**	**301**	**302**
XII, 7	19.0	22	29	39	**58**	**75**	**87**	**104**	**121**	**133**	**153**	**165**	**175**	**185**	**189**	....	**193**	**211**	**217**	**230**	**237**	**250**	**258**	**278**	**287**	**303**	**321**	**331**
XIII, 5	32.5	40	**50**	**65**	**79**	87	....	127	150	173	190	215	226	**257**	266	**290**	**297**	**314**	**321**	**332**	**344**	**340**	**355**	**369**	**379**	**383**	**390**	**391**
Average	21.1	26	33	44	**59**	**74**	**83**	**102**	**117**	**130**	**144**	**156**	**166**	**176**	**188**	205	**206**	**222**	**231**	**238**	**247**	**255**	**264**	**272**	**276**	**284**	**297**	**303**
Abnormal Experimental Males (XII, 3; XIII, 3) or without Controls (IX, 6; X 3)																												
IX, 6	19.3	30	37	49	65	79	96	117	136	154	158	171	189	199	226	235	....	254	267	280	293	307	319	326	344	346	343	338
X, 3	21.6	31	39	54	68	79	91	115	139	158	168	199	223	248	243	261	290	299	290	287	312	324	321	324	322	312	347	357
XII, 3	20.6	24	28	40	51	62	75	94	109	121	136	146	161	163	167	....	170	177	181	187	187	187	189	192	201	207	197*	
XIII, 3	29.3	36	43	55	69	80	....	124	145	165	176	190	192	214	233	248	260	272	262	267	277	269	278	281	280	285	278	281†
Average of all experimental males	21.8	27	34	46	60	74	84	106	122	137	150	163	175	187	199	224	216	232	238	244	254	261	259	275	279	285	296	299
Control Males																												
III, 5	**17.7**	**24**	**33**	**45**	**60**	**78**	**86**	95	112	123	133	138	**146**	**154**	**165**	**178**	**168**	175	184	188	201	209	206	211	228	220	220	216
VIII, 9	**19.8**	**24**	**31**	**40**	**51**	61	**74**	**84**	**96**	**110**	**120**	**129**	**137**	**140**	**145**	**164**	**176**	**188**	**198**	**207**	**211**	**214**	**227**	227	230	238	235	237
XI, 7	23.8	31	36	46	59	74	94	109	117	135	148	157	168	182	191	201	205	209	220	217	228	232	237	242	235	223	254	269
XII, 8	**19.0**	**23**	**30**	**38**	**48**	62	**75**	**88**	**104**	108	120	128	139	140	153	....	175	184	187	191	198	203	210	216	220	226	223	231
XII, 2	**21.8**	**25**	29	43	58	65	79	91	106	117	126	143	151	164	178	....	189	207	213	214	226	223	228	232	233	237	237	237
XII, 9	**20.7**	**24**	**30**	**42**	57	70	84	95	111	122	136	150	160	162	174	....	186	194	194	193	202	205	214	220	225	226	220	224
XIII, 4	**33.0**	**42**	48	58	71	**91**	....	**140**	**162**	**187**	**208**	**231**	**234**	251	**274**	283	287	300	303	313	316	325	332	347	354	364	360	359
Average	**22.3**	**28**	**34**	**45**	58	72	82	100	115	129	142	154	162	170	183	207	198	208	214	218	226	230	236	242	246	248	250	253
Controls without Experimental Litter-Mates																												
II, 4	16.7	20	22	31	40	50	62	70	85	91	96	105	111	122	131	140	150	160	170	182	193	194	213	215	217	215	221	230
II, 5	17.9	23	30	41	53	68	84	95	110	121	138	160	176	192	203	211	220	225	233	240	250	252	252	276	270	273	280	285
XII, 4	16.4	20	28	37	49	61	60	72	88	98	103	116	125	130	145	....	149	162	162	161	167	167	172	182	188	191	188	192
XIII, 2	29.6	38	45	60	69	87	....	134	154	175	191	208	206	230	245	260	268	272	279	300	300	307	315	318	328	323	320	335
Average of all control males	21.5	27	33	44	56	70	70	98	113	126	138	151	159	170	182	205	198	207	213	218	227	230	237	244	266	248	251	256

* Peritoneum appeared abnormal. † Abnormal. Lost weight steadily after this to 220 days (169 gm.).

porary increase in weight in the females in Figure 2 was caused by pregnancy. The ensuing drop in weight was caused by delivery of a litter of young.

An examination of Table II shows that the seven experimental males were heavier than their controls at and after 100, 135, 15, 60, 30, 35, and 25 days of age, respectively. The average weight of the experimentals became greater than that of their controls at 35 days of age, but the difference did not become marked until about 100 days of age, when the difference averaged 12 gm. per animal. If the comparison includes all the animals in the table, the experimentals averaged more than all the controls at 25 days; but this figure means little because some of the extra four experimental and four control animals were not normal, or experimentals and controls did not come from the same litter. These animals are included for the sake of completeness and to avoid personal selection on the part of the authors. As in the case of the females, the increased growth of the pituitary injected males is greater after 100 days of age than before, although the results indicate some influence of the extract before this age, otherwise the crossing of the curves of growth at from 15 to 100 days would be difficult to explain.

Experimental animal VIII, 10, failed to keep up in growth with its control, VIII, 9 (which was 1.8 gm. heavier at 15 days), but caught up with it at 40 days, to lag behind again to 135 days, when it became and remained the heavier of the two. Autopsy showed nothing wrong with this animal, but it is not unlikely that it had been injured in the experiment in some way. The striking gain after about 100 days of age should be noted.

The growth in the males in two of the groups is shown in Figure 1 (5 vs. 7) and Figure 2 (5, 7, 1, vs. 2, 8, 9). In these graphs it is also evident that the greatest effect of the extract is the continuance of growth of the experimentals after the controls have passed their period of most rapid growth and their growth curves are plateauing. Yet the divergence of the curves of the experimentals and controls is often definite at an early age.

While the fact that each experimental animal having a control was heavier than its litter-mate control of the same sex at 150 days of age appeared significant in itself, yet it was decided to treat the

* Peritoneum appeared abnormal. † Abnormal. Lost weight steadily after this to 220 days (160 gm.).

data statistically. For the females, the actual difference divided by the error of the difference was 6.1, a significant figure. For the males,

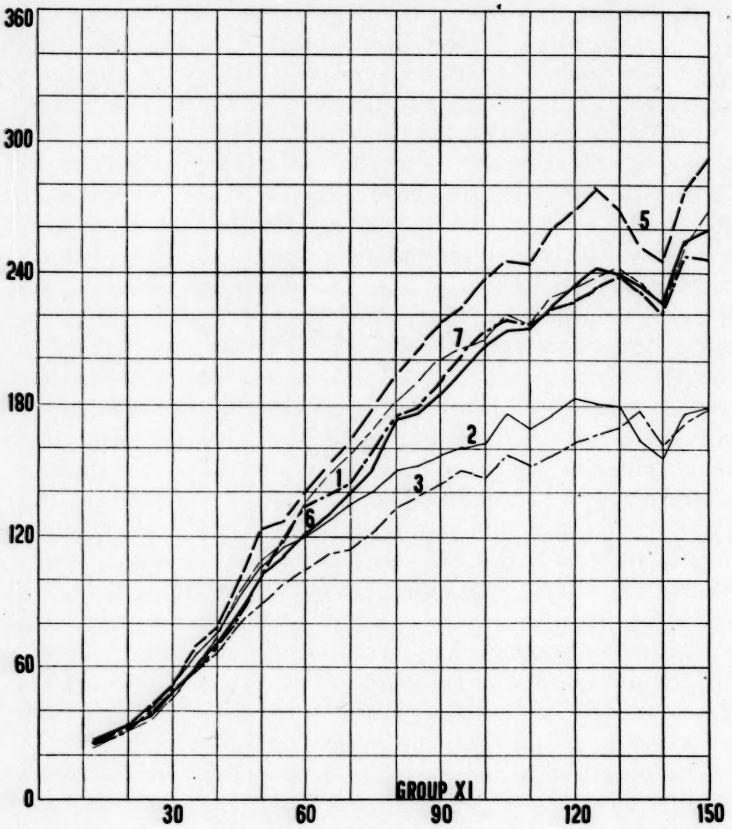

FIG. 1.—Growth curves of rats in Group XI. 5, experimental male; 7, control male; 1 and 6, experimental females; 2 and 3, control females; abscissas, days; ordinates, grams.

The drop in all the growth curves at 140 days was produced by a temporary decrease in amount of food given the rats by a new attendant.

this number was 3.1, which, if the males were considered alone, would not be significant. The fact that both experimental males and females with litter-mate controls as well as those not properly

controlled all showed the same response would make the data for both sexes more significant than if only one sex or only those with proper controls had been used.

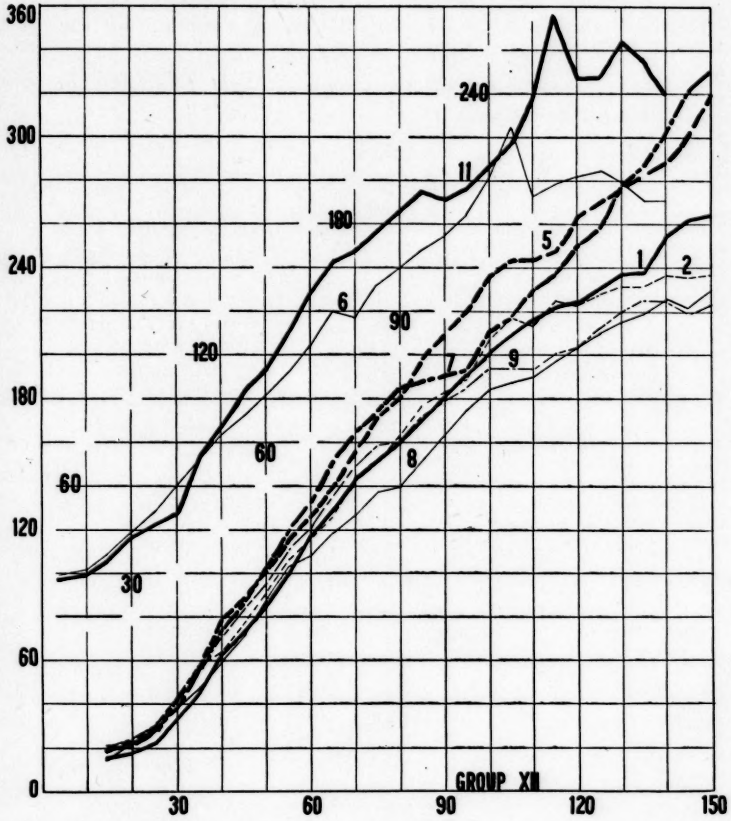

FIG. 2.—Growth curves of rats in Group XII. 5, 7, and 1, experimental males; 2, 8, and 9, control males; 11, experimental female; 6, control female; abscissas, days; ordinates, grams.

The curves of the females (6 and 11), accompanied by numbers applying to them, form an insert which is to be examined separately from the remainder of the figure.

From this figure are omitted an experimental male (3) which had an abnormally thick and rough peritoneum and a control male (4) which had slight adhesions of the viscera to the body wall.

The increased weight of the experimental animals was partly accounted for by a general increase in body growth, but the muscles and viscera appeared enlarged out of proportion to the remainder of the body. The abdominal muscles were especially heavy. Measurements of the leg bones (Table III) did not indicate that the increased weight of the experimental animals was produced primarily by an increase in bone length, for these bones averaged only slightly longer than in the controls.

TABLE III

MEASUREMENTS AND OBSERVATIONS AT AUTOPSY AT 150 DAYS

FEMALES

Group and No.	Length (Mm.)			Parasitism by Tapeworms*	Uterus†	Group and No.	Length (Mm.)			Parasitism by Tapeworms*	Uterus†
	Body	Tail (if Whole)	Leg				Body	Tail (if Whole)	Leg		
	Experimental Females						Control Females				
III, 7	205	Short	102	3	3	III, 6	191	Short	95	0	4
VII, 2	202	Short	105	3	2	VII, 4	197	Short	97	2	3
VII, 7	203	177	97	3	3	VII, 11**	.....	.....	.....	0	4
X, 6	211	192	98	2	1	X, 7	211	189	105	1	2
XI, 1	205	180	98	0	1	XI, 3	185	158	96	0	1
XI, 6	212	175	100	3	1	XI, 2	190	160	91	4	1
XII, 11	205	180	101	3	4	XII, 6	200	159	98	4	4
Average	206	181	100	2.4	2.1	Average	196	166	97	1.6	2.7

Group and No.	Length (Mm.)			Parasitism by Tapeworms*	Uterus†
	Body	Tail (if Whole)	Leg		
Experimental Females without Controls					
II, 3	217	159	98	3	2
IX, 2	215	205	107	1	2
IX, 5	212	201	105	0	2
X, 2	206	194	102	2	2
X, 5	198	185	100	0	2
Average of all experimental females	208	185	101	1.9	2.1

TABLE III—*Continued*

Group and No.	Length (Mm.)			Parasitism by Tapeworms*	Testes Volume (Cc.)	
	Body	Tail (if Whole)	Leg		Right	Left
Experimental Males						
III, 10....	221	Short	110	0	0.86	0.75
VIII, 10..	206	193	103	1	2.02	1.81
XI, 5.....	218	184	106	0	1.78	1.50
XII, 1....	205	180	101	3	1.29	2.28
XII, 5....	239	176	105	3	2.40	2.40
XII, 7....	232	181	110	2	.97	.95
Average	220	182	106	1.5	1.55	1.62
Experimental Males, Abnormal or without Controls						
IX, 6.....	228	206	109	0	1.71	1.71
X, 3.....	232	205	101	0	1.55	1.54
XII, 3††..	210	159	102	0	1.01	1.37
Average of all experimental males	221	186	105	1.0	1.51	1.59
					1.64	

Group and No.	Length (Mm.)			Parasitism by Tapeworms*	Testes Volume (Cc.)	
	Body	Tail (if Whole)	Leg		Right	Left
Control Males						
III, 5...	200	Short	113	0	....	....
VIII, 9..	213	169	101	3	1.48	0.94
XI, 7...	226	170	98	1	1.78	1.78
XII, 8...	212	159	102	1	1.58	1.44
XII, 2...	222	167	103	2	1.98	1.93
XII, 9...	212	166	101	3	1.67	1.84
Average	214	166	103	1.7	1.70	1.59
Control Males without Corresponding Experimentals						
II, 4....	215	Short	97	3	....	....
II, 5....	218	Short	95	3	....	....
XII, 4††	205	170	94	3	1.54	1.89
Average of all control males..	214	167	101	2.1	1.67	1.64
					1.66	

* None = 0; light = 1; medium = 2; heavy = 3.

† Slender = 1; slender, red = 2; thick = 3, thick, red = 4.

** Dead at full term.

†† Peritoneum abnormal or adhesions. In other animals it was normal.

The body lengths averaged about 10 and 6 mm. greater in the experimental females and males, respectively, than in the controls, and the leg measurements averaged 3 mm. greater for the experimental females and males than their controls; but statistical treatment of the lengths of the females showed no significance to these differences.

EFFECTS OF THE EXTRACT UPON THE FEMALE GENITAL SYSTEM

In the young female experimental rats the opening of the vagina occurred about the same time that it was found among the control females. Inhibition of reproduction was seen in the injected animals, only two litters being produced among the thirteen females, as

opposed to three litters among seven controls. Like the females, the males were not completely sterile, for one of the two litters mentioned came from cages containing only treated rats.

Macroscopic observations at autopsy at 150 days showed that the ovaries of the experimental females were larger than those of the controls, including those which had been pregnant. Microscopic study of the ovaries showed that this increased size was due to the presence of a large number of corpora lutea in treated females, ten or more showing in many sections as opposed to three or four somewhat larger ones in sections of control ovaries.

The scarcity of normal follicles and the presence of degenerating egg cells both in follicles and in corpora lutea led to a further study of possible transition stages from the follicle to the corpus luteum in the pituitary injected females. The smaller follicles were not as abundant as in the control ovaries, and some of them showed an irregular degenerating egg. Few of the larger follicles were normal. One ovum in the discus proligerus was in an anaphase stage of the first maturation division. In many the discus proligerus had disappeared and the egg, partially divided into three or four parts, was seen in the fibrous tissue within the follicular cavity. Scattered throughout most of the partially divided ova were small dark bodies which appeared to be nucleoli, since many of them were within light-colored circular areas resembling egg nuclei. In such follicles the stratum granulosum was being replaced by lutein cells. Later stages showed the lutein cells from half-closing to completely closing what had been the follicular cavity. In many of these the ovum was seen. It was usually partly or completely divided into about two to four cells each, with scattered nucleoli, some of which appeared to be in nuclei much as in the ova in the atretic follicles.

The uterus of the experimental animal had not increased in size or undergone any development different from that found in the control females which were not pregnant (Table III).

EFFECT UPON THE MALE GENITAL ORGANS

While no difference in size of the scrotum was noted, it appeared that the controls developed a scrotum earlier than the experimental animals. Other parts of the genital system showed no difference

in size in the two kinds of animals. Measurements of length, width, and thickness of both testes and the products of these were tabulated to give an indication of the relative volumes of the testes. The average of the products for the experimental animals was 1.55 cc. (right) and 1.62 cc. (left) and for the controls 1.70 cc. (right) and 1.59 cc. (left), measurements which showed no significant effect of the extract upon the size of the testes at 150 days of age.

POTENCY OF EXTRACT AS PREPARED

The extract prepared from glands which had been removed from cattle about 24 hours showed that it was potent because increased weight in the experimental animals was produced by the daily injections, no difference being noted in the effect of different batches. That the principle which affects the sex organs was also present was shown by the histological picture of the ovaries. From handling the material week after week, it can be said that to keep an extract usable it must be kept cold; and the filtration process must not be delayed too long, as the fluid held in the filter paper with the extract may putrefy and refiltering will not help it. Sodium benzoate is valuable in extracts which are over a week old, as it tends to cause any new precipitate to settle out. Precipitates which form in extracts that lack the sodium benzoate tend to remain in suspension in the liquid. Refiltering of unputrefied extracts in which precipitates form aids in the prolongation of the usefulness of the extract. Such refiltered extracts produce results no less marked than the fresh extract.

DISCUSSION

The absence of a significant increase in bone growth in the treated rats of this experiment is not contradictory to the work of Evans (1924), in whose experimental rats there was an overgrowth of the skeleton, of most of the viscera, and also an accumulation of fat, because most of the skeletal overgrowth could have taken place in his experimental animals after the 150th day, the time at which our experiment stopped.

Apparently an excess of the anterior pituitary growth hormone is not strikingly effective in the production of the long bones before the 150th day but tends to produce a general increase in growth.

Excessive skeletal growth beyond this time appears to be made possible by a delay in the complete ossification of the long bones in the anterior pituitary injected rats. This ossification occurs at about 150 days in the normal female rat and at about 200 days in the normal male. An examination of Table III may raise the question if parasitism by tapeworms may have been responsible for the small size of some of the rats. However, a study of Table III reveals the fact that parasitism was heavier in the group of seven experimental males than in their controls, and practically equal in the group of six female experimentals and six controls; so parasitism is not a determining factor in these experiments.

That lutein tissue formation is caused by a hormone distinct from the growth hormone was suggested by Evans (1924), whose extracts treated in a certain way did not affect the female sex organs but still produced body growth. Our work lends some support to this idea, since two injected female rats became pregnant and yet were larger than the uninjected controls, indicating that lutein formation and absence of normal ovulation are not always associated with the growth hormone.[1]

[1] Since this paper was written (July, 1928) our attention has been called to new work by Zondek and Aschheim (Klin. Wchnschr., 1928, VII, 831) and by Evans and Simpson (Jour. Amer. Med. Assn., 1928, XCI, 1337) showing that a maturity-producing hormone can be extracted from the anterior pituitary body. Evans and Simpson give evidences that the growth hormone extracted in alkali and subsequently neutralized inhibits the effect of the maturity-producing hormone. This would explain the usual lack of reproduction by our pituitary injected rats. In the two cases of reproduction (Table I) one would then have to assume that not enough growth hormone was received to inhibit ovulation, which may be true since these two females were a little below the average weight of the treated rats, although they were heavier than the control rats.

Maturity production in the transplant method used by Smith (1927*c*) is possible, according to Evans and Simpson, because the maturity-producing hormone is readily absorbed from transplants but the antagonistic growth hormone is absorbed well only from extracts.

It appears strange, however, that the growth hormone should both inhibit maturity production and stimulate formation of lutein tissue, for lutein tissue formation is normally associated with sexual maturity. In our experiments it was found that before lutein tissue was formed, abnormal follicles were developed, some of them up to near the point at which ovulation should occur, but before ovulation could take place the egg was hemmed in by lutein cells. Further evidence that lutein formation may be an exaggerated effect of the maturity-producing hormone may perhaps be seen in the literature, such as (1) the very early production of lutein tissue in suckling rats by the injection method by Evans (1924), (2) the production of placentomata in injected rats by injur-

The cessation of growth at or shortly after sexual maturity in rats suggests the possibility that the growth principle of the anterior pituitary is lost at this time, but this does not seem likely because of the promotion of growth by injections of extracts from glands of animals which are no longer growing. Possibly ossification of the long bones is a process which cannot be further delayed by moderate secretion of the growth hormone, but can by excessive doses administered to the animal. Or, may growth stasis be caused by a new hormone developed at this time to counteract the growth principle of the pituitary? It would be desirable to know whether the follicular hormone could do this. It has been shown by Allen, Doisey, Frances, Gibson, Robertson, Colgate, Kountz, and Johnston (1924) to cause development of the oviduct, uterus, and vagina, apparently producing sexual maturity.

It seems strange that the extract which is strong enough to produce such marked effects on the ovaries has practically no effect on the testes of the rats, except possibly in one case. In the largest rat the testes were smallest. Possibly the male rat has a greater resistance to the sex principle, as it has to some poisons, like red squill, and greater doses are necessary to affect the testes than will produce a change in the ovaries.

CONCLUSIONS

1. Daily injections of bovine anterior pituitary extract accelerated the growth-rate of young rats.

2. Experimental female rats did not cease their rate of rapid growth as early as did their controls.

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ing the uterine mucosa, a result not obtained in uninjected virgin rats (Teel, 1926), and (3) the obtaining of histological pictures in the ovaries and uterus of the kind produced by injection methods by means of excessive transplantations, used in an effort to get maximum response to transplants (Engle, 1928). Particularly from Engle's results it might seem possible that lutein formation may be produced (under certain conditions such as different dosages, possibly larger dosages, or the presence of large quantities of an antagonistic growth hormone) by the maturity-producing hormone, which usually produces follicles and normal ovulation first and lutein tissue later. Furthermore, Evans and Simpson (1928, *loc. cit.*) found no lutein formation when both transplants (maturity hormone) and injections (growth hormone) were combined, so lutein formation is not necessarily bound up with the growth hormone. It would appear to the authors that if lutein formation is not caused by the maturity-producing hormone, there may be a third hormone in the anterior pituitary, a lutein-forming hormone.

3. Treated rats of each sex were significantly heavier (males only slightly so) but did not have significantly longer bones than saline-injected litter-mate controls of the same sex at 150 days of age.

4. Increase in weight was due to general growth and also to marked increase in muscular and visceral development.

5. The extract produced no change in the uterus or in the time of opening of the vagina but reduced fertility of the females and produced an increased size of the ovaries, owing to the development of a large number of corpora lutea.

6. Some follicles and several corpora lutea contained degenerating ova.

7. The development in the size of the testes was not affected by the injections of the extract.

8. Extracts prepared from glands which had been chilled for 24 hours were potent, as they affected the body growth and the sex organs of the treated animals.

LITERATURE CITED

ALLEN, B. M. 1928. The influence of different parts of the hypophysis upon size and growth of *Rana* tadpoles. Physiol. Zoöl., I, 153–57.

ALLEN, E.; COLGATE, C.; DOISEY, E.; FRANCES, B.; GIBSON, H.; JOHNSTON, C.; KOUNTZ, W.; and ROBERTSON, L. 1924. The hormone of the ovarian follicle, its action in test animals. Amer. Jour. Physiol., LXVIII, 138.

CLAIRMONT, P., and EHRLICH, H. 1909. Über Transplantation der Hypophyse in die Milz von Versuchstieren. Arch. f. klin. Chirur., LXXXIX, 596–808.

ENGLE, E. T. 1927. Gonadal stimulating hormone of the anterior pituitary and heterosexual ovarian grafts. Proc. Soc. Exp. Biol. and Med., XXV, 83–84.

———. 1928. The rôle of the anterior pituitary in compensatory ovarian hypertrophy. Anat. Rec., XXXVII, 275–87.

EVANS, H. M. 1924. The function of the anterior hypophysis. Philadelphia: Lippincott Co.

EVANS, H. M., and LONG, J. A. 1921. The effect of the anterior lobe administered intraperitoneally upon growth, maturity and oestrus cycles of the rat. Anat. Rec., XXI, 62–63.

———. 1922. Characteristic effect upon growth, oestrus and ovulation induced by the intraperitoneal administration of fresh anterior hypophysis substance. Proc. Natl. Acad. Science, Wash., VIII, 38–39.

EVANS, H. M., and SIMPSON, M. E. 1925. Characteristics of the anterior hypophyseal hormones. Anat. Rec., XXIX, 356.

———. 1926. Effects of the anterior hypophyseal extracts on the male. *Ibid.*, XXXII, 206.

———. 1927. Resemblance of littermates in the degree of response to anterior hypophyseal fluid. *Ibid.*, XXV, 36–37.

EXNER, A. 1910. Über Hypophysentransplantationen und die Wirkung dieser experimentellen Hypersekretion. Deutsch. Zeitschrift f. Chir., CVII, 172–82.

FLOWER, C. F., and EVANS, H. M. 1925. The repair of dwarfism following thyroidectomy by administration of anterior pituitary fluid. Anat. Rec. XXIX, 383.

ISCOVESCO, H. 1913. La physiologie du lobe antérieur de l'hypophyse. Le lipoide du lobe antérior. C. R. Soc. Biol. (Paris), LXXV, 450–51.

PUTNAM, T.; TEEL, H. M.; and BENEDICT, E. B. 1928. The preparation of a sterile active extract of the anterior pituitary with some notes on its effects. Amer. Jour. Physiol., LXXXIV, 157–64.

ROBERTSON, T. B., and RAY, L. A. 1925. Australian Jour. Exp. Biol. and Med. Science (Adelaide), II, 173–88. (Abstract in Endocrinology, XI [1927], 70.)

SCHAFER, E. A. 1909. Croonian lecture: The functions of the pituitary body. Proc. of Roy. Soc., Ser. B., LXXXI, 442–67.

———. 1911. Die Functionen des Gehirnanhanges (Hypophysi-cerebri). Berner Universitätschriften. H. S. (Nearly same as 1909 paper.)

SMITH, P. E. 1926. Ablation and transplantation of the hypophysis in the white rat. Anat. Rec., XXXII, 221.

———. 1927*a*. The experimental feeding of fresh anterior pituitary substance to the hypophysectomized rats. Amer. Jour. Physiol., LXXXI, 20–26.

———. 1927*b*. The disabilities caused by hypophysectomy and their repair. Jour. Amer. Med. Assoc., LXXXVIII, 158–61.

———. 1927*c*. The induction of precocious sexual maturity by pituitary homeotransplants. Amer. Jour. Physiol., LXXX, 114–25.

SMITH, P. E., and ENGLE, E. L. 1927. The rôle of the anterior pituitary in the development and regulation of the genital system. Amer. Jour. Anat., XL, 160–215.

TEEL, H. M. 1926. The effects of injecting anterior hypophyseal fluid on the production of placentomata in rats. Amer. Jour. Physiol., LXXIX, 184.

VAN WAGENEN, G. 1927. Weight and dimensional effects of anterior pituitary extract on gonadectomized male rats. Anat. Rec., XXXV, 51.

———. 1928. Hypophyseal extract and growth after castration. Amer. Jour. Physiol., LXXXIV, 461–67.

CONTROL OF SEX IN CLADOCERA. IV. RELATION BETWEEN THE RATE OF THE MOTHER'S DEVELOPMENT AND THE SEX OF HER YOUNG[1]

(Two figures)

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IT WAS shown in a recent paper (Banta and Brown, 1929*a*) that the simple expedient of rearing a moderate number (ten) of parthenogenetic females of *Moina macrocopa* crowded together in the same bottle of culture medium caused them to produce about 42 per cent male offspring, whereas uncrowded mothers produced only female young. This result was interpreted as due to the accumulation of excretory products of the crowded mothers. In a second paper (Banta and Brown, 1929*b*) it was shown, in the course of attempts to duplicate the condition brought about by crowding, that the balance of these excretory products, as judged by its effect upon male production, was upset by a wide variety of treatments. In a third paper (Banta and Brown, 1929*c*) the time during which the sex of the forthcoming young was determined was found to be about 4 hours (at 20° C.) before the parthenogenetic eggs are laid. Since the introduction into semicrowded bottles of certain excretory substances and related products was unsuccessful as a means of inducing male production, our attention was directed toward a more complete study of the relations between the crowded environment and the animals themselves. It was noticed, in the course of the experiments covered in the papers mentioned, that there was some relationship between the time of release of a mother's first brood and the sex of the young in that brood. (Most broods are unisexual.)

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This observation directed our line of attack in attempting to unravel the effect which crowding might have on the females to make some of them produce males.

The present paper concerns data of experiments which have a bearing on the rate of the mother's development as a factor controlling or associated with the sex of her young. These data give in detail some work on different degrees of crowding, sex of young, and time of release of the young.

EXPERIMENTAL

Since mothers reared ten in a bottle were slower in developing than their uncrowded sisters, as judged by the time of release of their first clutch of young, it was felt that it would be of interest to increase experimentally the male percentage and note the relation of this to the time of release of the young by mothers so treated. The increased male percentages were induced by employing greater crowding of the mothers and consequently a greater concentration of the excretory products. Single broods were insufficient in numbers of individual females to be employed as test mothers in these experiments, so that several broods of the same size and age (to within a half-hour) were used. These mothers, while not sisters from the same brood, were granddaughters of the same individual. All the animals of the broods used in a single experiment were placed in the same watch glass and thoroughly mixed before they were transferred to the final bottles in the experiment. This precaution precluded any possible difference in the final sex ratios due to the use of more than one brood.

Table I gives a summary of eighteen such experiments, involving 5,866 young. The zero point in computing retardation was taken as the average time of release of the mothers in the first bottle to release in a given experiment. In some cases a few of the mothers put in the bottle died before the critical period for sex was reached by the majority of the animals in the bottle, and this accounts for a wider range in the different classes of crowding (e.g., 23–29) than would otherwise be the case, since only animals that actually reached maturity and hence were effective as crowders were counted, not the original number placed in the bottle. The bottles containing the

fewest mothers were the first to release in every case. It will be seen from the table that the male percentages increase with greater crowding and that the greater the delay in the release of the young, the higher the proportion of mothers which produce males. Other series of experiments similar to the four series here tabulated produced similar results.

DISCUSSION

In a former paper (Banta and Brown, 1929*a*) it was shown that the production of males by *Moina macrocopa* occurred when the animals either were crowded or were reared singly in a small amount

TABLE I

EXPERIMENTAL DATA ILLUSTRATING RESULTS OF THE DEGREE OF CROWDING UPON AMOUNT OF RETARDATION OF THE PRODUCTION OF YOUNG AND PERCENTAGE OF MALE YOUNG PRODUCED

No. of Experiments	No. of Mothers														
	2–4			7–9			12–17			23–29			30–36		
	Total Young Sexed	% ♂	Retardation in Hours	Total Young Sexed	% ♂	Retardation in Hours	Total Young Sexed	% ♂	Retardation in Hours	Total Young Sexed	% ♂	Retardation in Hours	Total Young Sexed	% ♂	Retardation in Hours
8.....	242	10.7	0	721	26.3	5.6	868	23.0	7.5	.....	.....	.....	.....	.....	.....
3.....	106	0	0	325	27.1	3.7	226	79.1	7.5	129	78.3	7	.....	.....	.....
4.....	243	0	0	475	22.3	6.6	718	48.5	8.2	908	65.6	11.4	.....	.....	.....
3.....	61	9.8	0	105	24.7	6.5	256	28.5	No record	.....	.....	.....	483	81.0	12.6
18.....	652	*4.9*	0	1,626	*25.3*	5.6	2,068	*35.8*	7.7	1,037	*68.1*	10.5	483	*81.0*	12.6

of culture medium. These facts, together with further experimental evidence—i.e., more males with heavier crowding; fewer males when the mothers were reared in the same amount of culture medium but in a shallow dish (thus permitting dissipation or oxidation of excretory products); reduced male percentage when the balance of the accumulated excretory products was disturbed by a number of various treatments; etc.—pointed to the accumulation of excretory products as the determining factor in these experiments. The experimental evidence now presented shows that the production of males by this animal is closely associated with a slower rate of development of the mothers producing them.

It is well known that the accumulation of an organism's own excretory products is a potent factor in retarding the development and limiting the growth of the organism. Semper's (1881) classical experiments with fresh-water snails produced results referable to this effect, though he proposed another explanation. He found that animals provided with optimum food conditions and reared in unchanged water in numbers or in individual containers grew in proportion to the amount of water per animal. De Varigny (1892) obtained similar results with snails and further found that the direct addition of the excreta of snails accentuated the unfavorable effect. Vernon (1902) found similar and very marked effects from rearing sea-urchin larvae crowded, or in water previously contaminated by other developing larvae or adults. Yung (1885) had similar results with rearing tadpoles. He also noted in addition, as De Varigny also found, that the amount of surface exposure of the water, presumably because of its effects upon oxidation of excretory products, was an important factor. Warren (1900) found with *Daphnia magna* that successive generations reared in unchanged water but presumably with plenty of food were slower in reaching the reproductive age and produced fewer offspring. Woodruff (1911) conducted carefully controlled experiments with *Paramecium* which led him to conclude that "the variation in the daily division rate in the different volumes of water is due to the excretion products of the paramecia themselves." Greenleaf (1926) working with *Paramecium* and other ciliates reached the same conclusion.

That the production of males of *Moina macrocopa* is associated with a retardation of the mother's development is shown by the fact that in bottles containing from two to thirty-six mothers the amount of retardation in the average time of release of mothers' first-broods is proportional to the number of mothers in a bottle (Fig. 1); and the percentages of males produced by these mothers is proportional to the amount of retardation (Fig. 2*A*). From this it follows (Fig. 2*B*) that the percentage of males increases in a linear order with the degree of crowding, i.e., with the concentration of excretory products in the culture water.

It is not possible to determine from a study of the data presented in this paper whether the slower rate of development of the mothers

is the causative factor in male production or is merely a concurrent phenomenon. Later papers in this series will give data on the experimental control of the rate of development by means other than crowding and the corresponding changes in the proportion of males. Later papers, also, will present evidence on the rate of development of female and male embryos and on the rate of development (as indicated by the time of egg-laying) of mothers which are to produce female and male embryos.

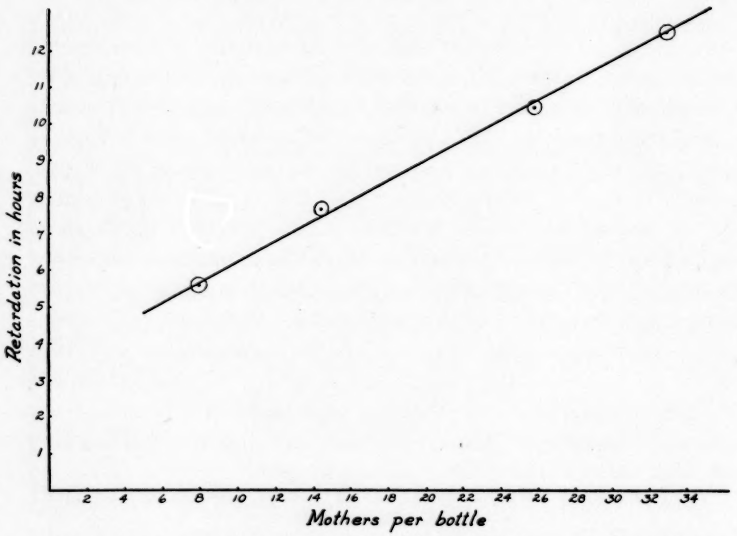

FIG. 1.—Diagram showing the relation between the numbers of mothers reared in a bottle and the amount of retardation in the production of the first clutch of young.

SUMMARY

1. The production of males is closely associated with, or caused by, a reduction in the rate of development of the mothers producing them. This retardation of the mothers is due in these experiments to the concentration of excretory products in crowded bottles.

2. The retardation of the mothers' development is proportional to the degree of crowding.

3. The percentage of males produced is proportional to the amount of crowding and to the retardation of the mothers' development.

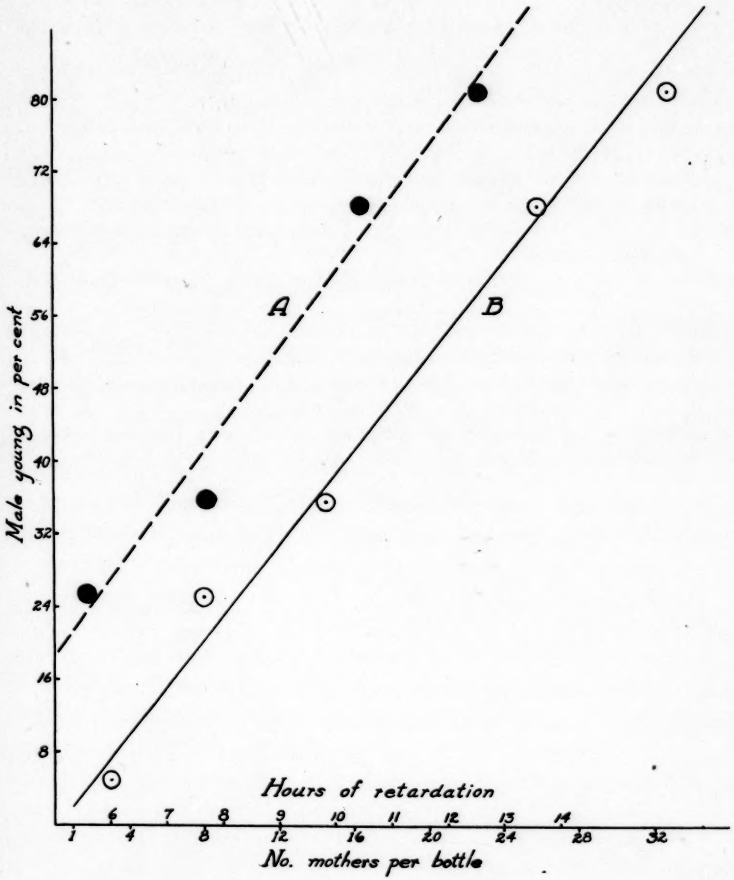

FIG. 2.—Diagram showing the relation (*A*) between the amount of retardation of the production of young and the percentage of males produced, and (*B*) between the number of mothers per bottle and the percentage of males produced.

LITERATURE CITED

BANTA, A. M., and BROWN, L. A. 1929*a*. Control of sex in *Cladocera*. I. Crowding the mothers as a means of controlling male production. Physiol. Zoöl., II, 80.

———. 1929*b*. Control of sex in *Cladocera*. II. The unstable nature of the excretory products involved in male production. *Ibid.*, II, 93.

———. 1929*c*. Control of sex in *Cladocera*. III. Location of the critical period for control of sex. Proc. Nat. Acad. Sci., XV, 71.

DE VARIGNY, H. 1892. Experimental evolution. New York: Macmillan Co.

GREENLEAF, W. E. 1926. The influence of volume of culture medium and cell proximity on the rate of reproduction of infusoria. Jour. Exp. Zoöl., XLVI, 143.

SEMPER, K. 1881. Animal life as affected by the natural conditions of existence. New York: Appleton & Co.

VERNON, H. M. 1902. Variation in animals and plants. New York: Holt & Co.

WARREN, E. 1899. On the reaction of *Daphnia magna* (Straus) to certain changes in its environment. Quar. Jour. Mic. Sci., XLII, 199.

WOODRUFF, L. L. 1911. The effects of excretion products of *Paramecium* on its rate of reproduction. Jour. Exp. Zoöl., X, 557.

YUNG, E. 1885. De l'influence des variations du milieu physico-chimique sur le développement des animaux. Arch. des Sci. Phys. et Nat., XIV, 502.

CONTROL OF SEX IN CLADOCERA. V. EXPERIMENTALLY ACCELERATED DEVELOPMENT OF MOTHERS AND SEX OF YOUNG; MAMMALIAN ENDOCRINE SUBSTANCES WITHOUT SPECIFIC EFFECT ON CLADOCERANS[1]

(One figure)

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A HIGHER rate of development of a female *Moina macrocopa* is associated, either causally or concurrently, with the production of female young; and a lower rate is associated with male production (Banta and Brown, 1929*d*). This was shown by the relation that prevails between the time of release of first broods of young by mothers and their sex and by the linear relation that exists between the amount of retardation in development of mothers reared in progressively greater numbers in the same amount of culture water and the sex ratio of their young. The mothers were retarded by the accumulation of excretory products (Banta and Brown, 1929*a* and 1929*b*). If the rate of development is the operating factor in the determination of sex of the forthcoming young, then experimentally changing the rate of development should change the sex ratio of the young.

There are many agencies which tend to stimulate the general metabolism and speed of development of animals, and many agencies, also, that depress metabolism and retard development. It is well known that light dosages of ethyl alcohol and of many other anesthetics may increase the rate of metabolism and of development. As illustrations of effects of ethyl alcohol among plants and invertebrates: Calkins and Lieb (1902) and Woodruff (1908) obtained in-

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creased division rate in *Paramecium* when the animals were subjected to 0.04–0.16 per cent concentrations; Czapek (1913) cites a number of cases of such effects on plants and animals; Medes and McClendon (1920) with 1–6 per cent increased the oxygen consumption of *Elodea* 20 to nearly 300 per cent, and with the lower concentration found that the effects might increase for many hours and persist for 2 or 3 days; and Buchanan (1922, 1923) with about 0.25 per cent obtained increased head regeneration in planarians and also found that both whole animals and regenerating parts showed increased oxygen consumption (to a maximum of 700 per cent increase) when subjected to about 0.50 per cent concentration. Not all work with ethyl alcohol in light dosages has shown this effect, but it has been so frequently obtained as to be accredited as a general effect.

The effects of various endocrine substances upon vertebrates are well known, but their influence upon invertebrates is a mooted question which will be briefly discussed at the end of this paper. One earlier report may be mentioned here inasmuch as it influenced our decision to use endocrine gland products with our material. Van Herwerden (1923*a*), working with a cladoceran, *Daphnia pulex*, had reported a marked increase in rate of growth and in reproductive vigor as a result of using dried adrenal cortex with her cultures.

The data presented in this paper were obtained in an effort to produce, by means of treatments with stimulating substances, acceleration of development of crowded *Moina macrocopa* mothers which normally are retarded by the crowding and produce about 42 per cent males among their offspring, and to learn if such acceleration would be accompanied by a reduction in male production. While not anticipated at the start, these experiments provided some information as to the possible specific effects of endocrine substances on this invertebrate. A brief discussion of these findings is given. The data obtained in experimentally retarding the rate of development of females of this same species of *Cladocera* by means other than crowding will be presented in later papers.

EXPERIMENTAL

The general procedure was to take for each experiment a large brood of young females (16–26) from a single mother, and for all of

a series of experiments conducted simultaneously to use granddaughters of the same individual. In each experiment the young females were divided equally between two bottles. One bottle was held as a control, which ordinarily, because of the crowding (Banta and Brown, 1929*a*), produced many males; the second bottle was treated with substances which might stimulate the animals to a slightly increased rate of development. The time of treatment was varied somewhat, but was usually during the third instar, and, further, during that part of the third instar in which the critical period for sex determination is located, i.e., approximately 4 hours before the eggs are laid (Banta and Brown, 1929*c*). Each series of experiments consisted usually of five to twenty separate experiments. In the tables of data that follow, each such group of experiments, running concurrently under the same conditions of food, temperature, light, etc., and having, as it is possible to have only in a parthenogenetically reproducing form, animals which are genotypically identical, is summarized.

The difference in the rate of development of the control and test mothers was obtained at the time of release of the first brood of young by counting and removing from the bottles at half-hour or hour intervals the mothers that had released their broods. In a bottle containing thirteen mothers, a typical record of the mothers releasing at successive hours is 0, 1, 5, 4, 2, 1, 0; in a bottle having seventeen mothers, 0, 2, 6, 5, 2, 1, 1, 0. The mean of such a distribution array was taken as the time of release of that particular bottle. After all the mothers had released, the young were sexed, and times of release and the percentages of males produced for the control and test bottles were compared.

A difficulty involved in these experiments with alcohol and gland substances was that these substances produced secondary changes in the culture medium. Not infrequently these changes were manifest in a cloudiness or other visibly different appearance of the culture medium in the treated bottles. These changes were not evident until several hours after the treatments were applied. Since in general the treatments began only a few hours before the critical period, these secondary changes, for the most part, occurred after the sex of the young to be produced was fixed. But such changes

occasionally killed many of the test mothers, thus causing the elimination of an experiment and in other cases obviously delaying the release of young by the test mothers. The rather wide differences in amount of acceleration of development, as indicated by the time of release of young, in the different experiments are due principally to this factor. To avoid fouling of the culture medium by the treatments, some of the series of mothers from the start of the experiment (second or early third instar) were placed in clear pond water (or dilute food) instead of the usual manure solution and were kept in the pond water until the treatment of the test bottles was completed and the mothers had laid their eggs; they then were transferred to the regular culture medium. While this procedure eliminated the fouling of the culture bottles following the treatments, it also resulted in fewer young and in a lessened accumulation of excretory products, together with a consequent reduction in male percentage in both tests and controls.

ETHYL ALCOHOL

The first stimulating agent used was ethyl alcohol. Treatments sufficient to make the culture medium 0.2–0.6 per cent alcohol at the time of treatment were generally employed, usually 2–6 hours before the critical period. No effort was made to determine the extent of losses of the alcohol content by evaporation or by breaking down in our culture medium, both of which factors were operative. Treatments sufficient to make the culture medium more than 1 per cent alcohol resulted in the death of many of the animals and production of young by a very limited number of the survivors. The results of eighty-eight experiments are given in Table I. In these experiments the average male percentage of the untreated control (given distilled water or pond water instead of alcohol) was 36.1; the alcohol-treated tests gave but 5.5 per cent males, about one-seventh as large a percentage as the controls. It will be seen from the table that in every one of the thirteen groups of experiments the male percentage of the treated bottles was less than that shown by the controls. In many cases the release record was not obtained but for twenty-six experiments we have complete data. In each of these groups the alcohol-treated bottles released their young before the controls. Taking these latter twenty-six experiments together,

the treated bottles not only showed a marked decrease in male production (about three-tenths that of their controls), but they released their young on the average 2 hours and 42 minutes before the controls. This acceleration (2.7 hours) represents about 6 per cent of the entire time between the treatment of the test bottles and the release of the young at room temperature (about 22° C.).

TABLE I

EXPERIMENTS WITH ETHYL ALCOHOL*

No. of Experiments	Untreated Controls		Alcohol-Treated Tests		
	Total Young Sexed	% ♂	Total Young Sexed	% ♂	Acceleration in Hours
10.....	1,162	37.2	1,087	0.2	no record
4.....	170	4.7	258	0.0	no record
6.....	718	65.6	512	7.8	no record
4.....	198	11.6	198	0.0	no record
11.....	1,050	5.3	1,274	0.0	no record
11.....	1,076	15.8	1,122	0.5	no record
7.....	699	18.3	729	0.0	no record
2.....	71	15.5	37	0.0	no record
7.....	323	33.1	184	15.2	no record
5.....	348	22.4	335	3.6	0.5
3.....	287	62.7	273	44.7	2.1
10.....	865	45.3	621	9.3	3.7
8.....	803	80.2	813	17.7	2.9
Totals					
88.....	7,770	36.1	7,443	5.5	.....
Totals for Those Having Record on Release					
26.....	2,303	56.6	2,042	16.4	2.7

* Summary of experiments with ethyl alcohol as an agent to accelerate developmental rate and thus reduce male production in crowded bottles. Controls and tests were equally crowded.

ADRENAL CORTEX

Other extensive series of experiments involved the use of the adrenal cortex of the ox. This substance, as were the other glands and the muscle tissue, was prepared for use by soaking 250 mgm. of the desiccated powdered gland[1] in 10 cc. of distilled water for

[1] Some of the gland materials used were freshly prepared products obtained from Dr. Oscar Riddle of the Department of Genetics of the Carnegie Institution of Washington. Others, including specially prepared desiccated muscle of the ox, were furnished by G. W. Carnrick Co., New York City. We tender our best thanks for these courtesies.

4–6 hours at room temperature. This was filtered and the filtrate used immediately. A treatment with the gland and muscle filtrate consisted of from 0.2 to 1.25 cc., usually about 0.6 cc. in amount, to 75 cc. of the culture medium, administered as always, shortly before the critical period. Table II shows that this treatment with the prepared filtrate of the adrenal cortex had the desired effects (1) in accelerating the developmental rate of the treated animals to the time of releasing their first brood young by 48 minutes on the average, and (2) in reducing the male percentage from 23.97 to 5.21 per cent, about one-fifth that of the controls.

THYROID

Thyroid gland also was used (see Table II) and had a very similar effect, since it caused the treated mothers to release their young on the average 90 minutes before the controls and reduced the male percentage to 4.02. The greater apparent acceleration in the release of these thyroid-treated mothers may be due to the paucity of experiments in which the release record was obtained. Certainly one observing the animals during the course of the experiments noted no difference between the animals treated with adrenal cortex and those treated with thyroid. Several experiments with thymus gland, although not here tabulated, gave a result quite similar to that for the other two glands.[1]

DESICCATED MUSCLE

Since the effect of these glands on the rate of development and on the male percentage was rather pronounced and uniform for the different gland substances used, control series of experiments using dried muscle of the ox were run to see if the results obtained were specific effects of the glandular materials used. The results obtained with muscle are shown to the right in Table II. It is quite evident that the muscle filtrate gave the same general reduction in male percentage as the gland filtrates, namely 5.60 per cent males as compared with 23.97 per cent in the controls, and at the same time caused an acceleration in the time of release of the young of 48 minutes on the average.

[1] From eighteen experiments with thymus-treated mothers, the 1,988 sexed young were all females; while of the 2,462 control young, 21 per cent were males. Thyroid-treated mothers in the same experiments likewise produced only female young.

TABLE II

TABLE II

EXPERIMENTS WITH GLAND AND MUSCLE-TISSUE FILTRATES*

No. of Experiments	Untreated Control		Treatment								
			Adrenal Cortex			Thyroid			Muscle		
	Total Young Sexed	% ♂	Total Young Sexed	% ♂	Release Record in Hours	Total Young Sexed	% ♂	Release Record in Hours	Total Young Sexed	% ♂	Release Record in Hours
8	853	33.4	828	0.0	4.1 earlier						
7	781	8.8	679	0.0	0.5 earlier						
17	1,804	21.4	1,766	0.6	0.8 earlier						
13	1,316	13.1	1,257	1.0	earlier						
5	551	14.2				538	1.5	2.0 earlier			
9	807	48.3				1,133	7.9	2.5 earlier			
10	852	26.9				739	3.0	no record			
8	733	44.2	831	12.8	3.5 earlier						
5	324	24.4	298	0.7	no record						
5	550	7.8				538	2.2	1.0± earlier			
5	371	14.4	550	5.8	2.0± earlier						
9	1,002	14.5				1,005	2.2	earlier			
7	753	74.2				813	0.8	2.5 earlier			
9	1,040	32.9				1,066	1.6	no record			
4	434	25.1				435	0.0	earlier			
13	1,433	30.4				1,420	0.0	no record			
3	334	32.6	88	15.9	no record						
8	786	0.6				700	2.9	earlier	583	0.0	sl. earlier
13	1,498	4.2				1,237	0.0	no record	1,505	1.0	no record
14	1,386	39.7				829	19.3	no record	918	12.7	no record
9			166	1.2					465	2.8	3.0± earlier
9			264	10.5					406	15.5	4.0± earlier
7	319	22.0	823	3.3	0.7 earlier				798	6.5	1.7 earlier
17			2,103	0.0					2,184	1.0	no difference
1	92	10.8	102	0.0	0.9 earlier				101	0.0	2.7 earlier
16			1,814	8.2					2,013	0.6	earlier
12	1,428	37.0	1,248	3.5	sl. later				1,244	3.2	sl. earlier
7	637	17.7	496	0.0	0.7 later				628	0.1	0.8 earlier
16	1,356	20.6	1,279	9.6	0.4 earlier				1,408	15.0	0.5 earlier
6	658	26.1							647	9.0	2.2 earlier
6	724	8.8	670	1.1	3.0 earlier						
20	2,172	14.0	1,391	21.8	0.8 later	1,455	8.4	0.8 earlier	1,517	13.4	0.3 earlier
298	24,994	23.97	16,653	5.21	0.8 earlier	11,908	4.02	1.5 earlier	14,417	5.60	0.8 earlier

* These substances were used as agents to accelerate developmental rate and thus reduce male production in crowded bottles. Controls and tests were equally crowded.

The male production by the crowded controls in these experiments was in general below the average for such degree of crowding, owing to changing the animals to a different culture medium during the experiments.

DISCUSSION

The chief point brought out by the data presented in the two tables is the fact that the rate of development of a female appears to regulate the course of sex-determination of the parthenogenetic eggs of her first brood. It was shown (Banta and Brown, 1929*d*) that animals in a crowded bottle, which normally produce many males, release their young later than animals not crowded, which produce only females. The use of alcohol or of certain endocrine gland and muscle filtrates tends to accelerate the rate of development and counteract this crowding effect, thereby causing the mothers to produce fewer males.

The male percentage produced by the crowded controls of the experiments here recorded is smaller than that shown in our earlier papers (Banta and Brown, 1929*a*, *b*, *c*, *d*). This discrepancy between the data for the percentage of males produced by the different degrees of crowding as given in those papers and the data given in the present paper is due to the fact that in the former experiments the animals were subjected to a male-producing environment (accumulation of excretory products) which was developing for the entire time from the first instar until after the critical period for sex control and to the subsequent release of young; while in the latter case, as already mentioned, due to the change in the medium (second or early third instar), the crowded situation was developing for only a limited portion of this time. This limited treatment and the transfer of animals from one bottle to another, from food to pond water or to dilute food, accounts for the smaller average male percentage prevailing for the control in these experiments than was previously found (42 per cent) in normally crowded bottles.

It should be emphasized that in the experiments with both the alcohol and with the dried endocrine glands and muscle the actual treatment with these substances occupied but a short delimited portion of the entire life of the mothers from birth to the production of the first brood of young. At 20° C. this entire time is about 114 hours, while the animals were usually subjected to the treatment for 24 hours or less, during which the animals passed through the critical period for sex-control (Banta and Brown, 1929*c*). The lack of agreement in some series of experiments between the male per-

centage and the time of release was due to the adverse cultural conditions developing in some of the treated bottles after the critical period and a delay in restoring the animals to good food. While the lack of transfer to good food could not (after the critical period) alter the proportion of males produced, it often did have a pronounced effect on the rate of development of the mothers following the time when the sex of the young is fixed.

It is interesting to note in this connection that in several cases, perhaps due to some irregularity in the treatment and to unusual cultural conditions, the treated bottles released *later* than the controls, and in a few cases also produced a *higher* proportion of males. A particular case that illustrates this point is the last group, a rather large group of twenty experiments, at the bottom of Table II. The time of release was obtained and the sex of the young determined with the usual care. Disregarding the type of treatment, when the time of release is plotted against the percentage of males produced (Fig. 1) a linear relationship is obtained. This indicates that the rate of the mothers' development during the critical period (regardless of the treatment employed) is a measure of the effectiveness of the male-influencing situation. Obviously in this case unusual cultural conditions were effective during the critical period for sex control.

While the influence of the thyroid hormone and other endocrine gland hormones on the growth and differentiation of vertebrates is quite definite and well known, the effects of administering vertebrate endocrine products to invertebrates are still uncertain. Any evidence to be had in this direction would seem, then, to be of interest. The data presented in this paper, while not gathered with this problem in mind, have a bearing on the question of the specific action of the glands of internal secretion on a group of invertebrates.

The first extensive work on the effect of the thyroid hormone in the lower animals was upon Protozoa, chiefly *Paramecium*. The earlier workers (Nowikoff, 1908; Shumway, 1914, 1917; Budington and Harvey, 1915) concluded from experiments in which various fresh and dried gland products were used with cultures of *Paramecium*, that the division rate of this protozoan was significantly increased. More recent work, particularly that of Woodruff and

Swingle (1924) and Ball (1925) tends to show that thyroid and other endocrine materials completely lack properties that accelerate cell division. Woodruff and Swingle had the advantage in having available thyroxin, the use of which avoids the very probable changes in the content of the thyroid-fed cultures. They found that it operated to retard rate of division. Torrey (1928) states that thyroxin

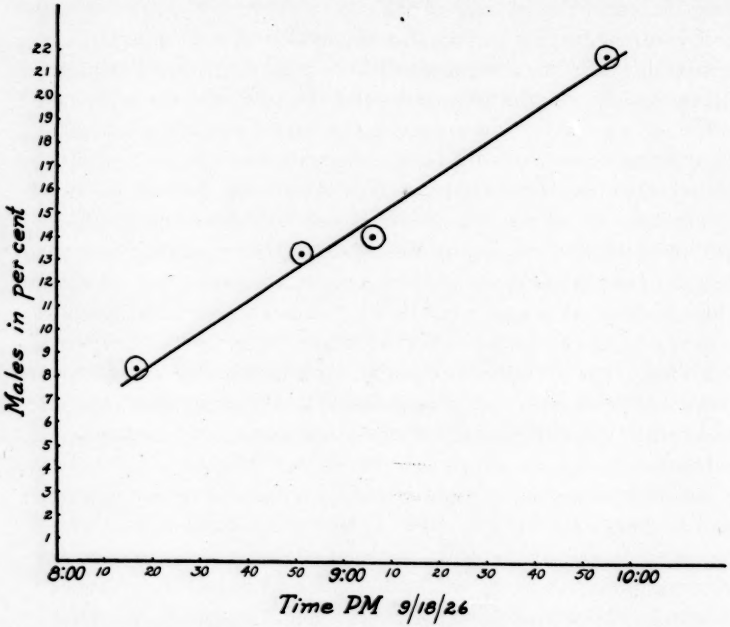

FIG. 1.—Graph for a single series of experiments (Expts. 2557–76), showing relation between retardation and male production, regardless of type of treatment—thyroid, muscle, control, and adrenal cortex in order from left to right.

retards both cleavage rate and differentiation in the sea urchin and the ascidian with which he worked.

The effect of thyroid on insect growth and metamorphosis is also in doubt. Kunkel (1918) fed the larvae of flesh flies fresh thyroid gland and used fresh muscle as a control. He found that thyroid treatment tends to hasten the onset of pupation; the size of the resulting pupae were somewhat smaller than the controls. Romeis

and Dobkiewicz (1920) used a species of blowfly as their experimental material. They found that the thyroid-fed larvae underwent pupation later than the control larvae and also that the pupae were slightly heavier. Kopec (1926), using a moth, *Lymantria dispar*, found no change in the duration of the larval or pupal periods and found that the only effect was a distinct diminution in the weight of the chrysalids of thyroid-fed larvae. Other workers on insects likewise report conflicting results.

The cortex of the adrenal gland as a dried preparation has been used extensively by van Herwerden on the growth of the fresh-water snail, *Limnaea* (1923*b*), and on the growth and rapidity of attaining sexual maturity in the cladoceran, *Daphnia pulex* (1923*a*). In both instances she found that the animals fed with adrenal cortex or with extracts of this portion of the gland grew faster and were less subject to death caused by unfavorable cultural conditions. She also found in *D. pulex*, as we have observed for *M. macrocopa*, that the length of time to maturity is shortened by the feeding of filtrates of adrenal cortex. Van Herwerden used liver as a control in some experiments and no additional food in other controls.

In our experiments, dried endocrine glands, particularly thyroid, thymus, and the adrenal cortex, do not have a *specific* effect upon the time of release of young cladocerans. This is evident for two reasons: first, the similar effect, namely, earlier release of young, of all the different glands used (adrenal medulla and pituitary gland were used in addition to the three already mentioned); and second, the effect produced by the addition of filtrates of the various endocrine glands could be duplicated by the addition of a like amount of dried-muscle tissue filtrate.

Hankó (1912) tested solutions or suspensions of various substances on the time of molting of the isopod *Asellus aquaticus*. He found that the substances he used stood in the following relation to each other in regard to their effectiveness in accelerating the first molt of his animals following an operation for regeneration: hypophysis extract > glycogen > lecithin > control (pond water) > sodium chloride > ethyl alcohol.

We feel that the results obtained in our experiments, in the experiments of van Herwerden, and in the somewhat different experi-

ments of Hankó are probably due to an alteration in the food content of the culture medium in which the animals are reared. This change in the culture medium, at least in our cultures, is probably due to some indirect action on the bacterial flora that serves as the food material for the daphnids. Northrup (1917) has shown that certain animal tissues including the adrenal and thyroid are not adequate foods for larval *Drosophila* unless bacteria are present. So far as we know in the feeding of invertebrates with endocrine products, the results, in view of the lack of an adequate control in many cases, are explicable as due to changes in the kind or amount of food rather than as a specific effect of some endocrine hormone.

SUMMARY

1. Ethyl alcohol (light dosages), the filtrates of dried adrenal cortex, thyroid, thymus, and muscle tissue increase the rate of development of *Moina macrocopa* mothers.

2. This increased rate of development largely counteracts the normally retarding effect of crowding the animals and accompanies or acts to materially reduce the proportion of males produced by such mothers.

3. Inasmuch as a filtrate of dried muscle produces the same effect as adrenal cortex, thyroid, and probably thymus, the latter substances show no specific effect on the rate of development of this animal. The observed results are probably due to changes in the bacterial flora which serves as food for this organism.

LITERATURE CITED

BALL, G. H. 1925. Studies on *Paramecium*. I. Experiments on the action of various endocrine substances, of liver, and of glycogen on the division rate of *Paramecium*. Univ. of Calif. Pub. in Zoöl. XXVI, 353.

BANTA, A. M., and BROWN, L. A. 1929*a*. Control of sex in *Cladocera*. I. Crowding the mothers as a means of controlling male production, Physiol. Zoöl., II, 80.

———. 1929*b*. Control of sex in *Cladocera*. II. The unstable nature of the excretory products involved in male production. *Ibid.*, p. 93.

———. 1929*c*. Control of sex in *Cladocera*. III. Localization of the critical period for control of sex. Proc. Nat. Acad. Sci., XV, 71.

———. 1929*d*. Control of sex in *Cladocera*. IV. Relation between time of release and the sex of the young. Physiol. Zoöl., II, 302.

BUCHANAN, J. W., 1922. The control of head formation in *Planaria* by means of anesthetics. Jour. Exp. Zoöl., XXXVI, 1.

———. 1923. Weight changes and oxygen consumption during long exposure to dilute anesthetics. *Ibid.*, XXXVIII, 331.

BUDINGTON, R. A., and HARVEY, H. F. 1915. Division rate in ciliate Protozoa as influenced by thyroid constituents. Biol. Bull., XXVIII, 304.

CZAPEK, F. 1913. Biochemie der Pflanzen. Jena.

HANKÓ, B. 1912. Über den Einfluss einiger Lösungen auf die Häutung, Regeneration, und das Wachstum von *Asellus aquaticus*. Arch. Entw. d. Org., XXXIV, 477.

HERWERDEN, M. A. van. 1923*a*. Der Einfluss der Nebennierenrinde auf das Wachstum und die Fruchtbarkeit von *Dapnia pulex*. Arch. f. Entw. d. Org., XCVIII, 221.

———. 1923*b*. Der Einfluss von kleinen Quantitäten der Nebennierenrinde des Rindes auf das Wachstum der Süsswasserschnecke *Limnaea*. *Ibid.*, p. 505.

KOPEC, S. 1926. Is the insect metamorphosis influenced by thyroid feeding? Biol. Bull., L, 339.

KUNKEL, B. W. 1918. The effects of the ductless glands on the development of the flesh flies. Jour. Exp. Zoöl., XXVI, 255.

MEDES, G., and MCCLENDON, J. F. 1920. Effects of anesthetics on various cell activities. Jour. Biol. Chem., XLII, 541.

NORTHRUP, J. H. 1917. The rôle of yeast in the nutrition of an insect (*Drosophila*). *Ibid.*, XXX, 181.

NOWIKOFF, M. 1908. Die Wirkung des Schilddrüsenextrakts auf Ciliaten. Arch. f. Prot., XI, 309.

ROMEIS, B., and DOBKIEWICZ, L. von. 1920. Experimentelle Untersuchungen über die Wirkung von Wirbeltierhormonen auf Wirbellose. I. Der Einfluss der Schilddrüsenfütterung auf Entwicklung und Wachstum der Schmeissfliege (*Calliphora vomitoria*). Arch. Entw. d. Org., XLVII, 119.

SHUMWAY, W. 1914. The effect of thyroid on the division rate of *Paramecium*. Jour. Exp. Zoöl., XVII, 297.

———. 1917. The effects of a thyroid diet upon *Paramecium*. *Ibid.*, XXII, 529.

TORREY, H. B. 1928. Thyroxin as a depressant of cell division; its effect on the cleavage and early development of sea urchin and ascidian. Endocrinology, XII, 65.

WOODRUFF, L. L. 1908. Effects of alcohol on the life cycle of *Infusoria*. Biol. Bull., XV, 85.

WOODRUFF, L. L., and SWINGLE, W. W. 1924. The effects of thyroid and some other endocrine products on *Paramecium*. Amer. Jour. Physiol., LXIX, 21.

ASSOCIATIVE HYSTERESIS IN MARINE FLATWORMS

(Two figures)

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A LONG-STANDING question in the field of animal behavior is the distribution of associative memory in the animal kingdom. Among the invertebrates it has been demonstrated in many arthropods and some molluscs (Bohn, 1920, chap. xix; Holmes, 1923, chaps. ix, x). Jennings (1907) thought he had found a manifestation of it in starfish, but his results were later shown by Moore (1920) probably to be due to injury. Yerkes (1912) claimed to have demonstrated habit formation in the earthworm; but, since the conclusions were based on one individual alone, generalizations cannot be made. According to Holmes (1923, p. 181), there is no case in which the formation of associations is satisfactorily proved among the large assortment of animals grouped under the term "vermes." He also makes a similar statement for coelenterates and echinoderms. Using ability to learn as a criterion of associative memory, Loeb (1902, chap. i) has said that it can be shown that infusoria, coelenterates, and worms do not possess a trace of associative memory; that only certain species of animals possess it; and that the mechanism of associative memory can only begin to function after the brain has reached a certain stage of development, both ontogenetically and phylogenetically.

I. THE CONDITIONED RESPONSE

Previous attempts to produce associations in the lower forms have failed because the animals have been subjected to complicated tasks of a higher order which require trace reflexes, labyrinthine reflexes, or chain-reflexes to complete the responses; even the simplest type of maze-learning—all goal-seeking varieties of learning—involve such mechanisms on the part of the animal. The most feasible method to demonstrate the existence of associative memory

is the simple conditioned reflex method as developed by Pavlov (1927), because it is the least elaborate method known. Experiments carried out on a polyclad flatworm (*Leptoplana*) found under rocks along the shore of Monterey Bay indicate that this creature does possess, in a rudimentary form, associative memory, as demonstrated by this method.

This organism is photokinetic. When rocks are overturned, the worms begin to move, and continue until they reach a shaded area; when placed in a dish of sea water, they move as long as the rays from an electric light strike them. As a rule they are quiescent in the dark. The animal begins to creep by first thrusting a part of its anterior end forward, to be followed by the remainder of the body. If the advancing end is touched during creeping, the snout is retracted, resulting in a momentary pause, in turn followed by a turning to one side and continuation of creeping, or else a plunge forward and continuation of creeping. These reactions persist for a short time upon successive stimulations of the advancing end, a continuation in the original direction being more usual than a turn to one side. Sometimes the worm becomes highly irritable after it has been stopped a few times, and then the pauses become shorter; progression becomes more persistent; occasionally a minor number of individuals would swim for a few seconds during the first few exposures. Then movement is impeded only because the stick is in its way. There are great individual differences in responses to contact stimulation and also in light sensitivity. In other words, some individuals are much more active in resisting impediments to their progress—even to the extent of suffering injury at the anterior end. Since these animals begin active creeping when light impinges upon them, and pause when they strike an obstracle, we have at hand the possibility of establishing a condition of a very simple sort. I have therefore attempted to condition these worms against photokinesis by means of contact stimulation applied simultaneously with the light.

The apparatus consisted of a 300-watt electric lamp clamped to a standard and crossbar, which could be moved around on the cement table. The filament of the lamp was 25 cm. above the table top. The worms were kept in finger bowls, each dish containing sea

water to a depth of a little less than 1 cm. and but one individual. A match rounded off at one end was used for contact stimulation. All the experimenting was done in a dark room illuminated with red light. In order to work with one individual while several were "resting" from previous exposure, a shoe box painted black on the inside was placed over each "resting" dish. By this means as many as five individuals could be carried through a complete training series at a time. Throughout the experiments the water in each dish was changed but twice a day, inasmuch as these organisms will live in still sea water for many days, so long as the water is sufficiently shallow to allow ample diffusion of respiratory gases. Temperature of the water was maintained between 17° and 20° C.

Before being experimented upon, each worm was placed in a dish and left in the dark room for 12 hours. During the "training" process an individual was exposed to the light for 5 minutes and then allowed to remain in darkness for 30 minutes, until twenty-five exposures were made, except between the twentieth and twenty-first exposures, at which time the animals enjoyed peace for an hour while the experimenter ate dinner. The worms were not fed at all during the series of experiments, since they can live for weeks without food, without apparent deterioration.

A trial was conducted as follows: The lamp was placed above a covered dish and permitted to burn for 5 minutes. When first uncovered, the animal was always found motionless. At the first outward thrust of its snout in response to illumination, the tip was touched with the end of the stick, whereupon the snout was retracted. Each succeeding time the subject attempted to advance it was touched (or momentarily stopped), until the light was shut off. At the end of the 5 minutes a box was placed over the dish and the next dish was uncovered, and the light was placed above it, and so on. The same experimenter did the touching in all experiments.

Experiments were carried out on seventeen individuals. As the trials progressed, the number of touches required to induce the animals to remain motionless while being stimulated with light became less and less. The curve representing this decrease is shown in Figure 1, curve 1. During the first 5-minute exposures the animals were so active that as many as 272 touches in one instance were

administered without stopping the animal—the lowest number for any animal was 19 during the first exposure. In all instances but one, less touches were given during the second exposure; and only in two cases did the touches during a later exposure exceed those for

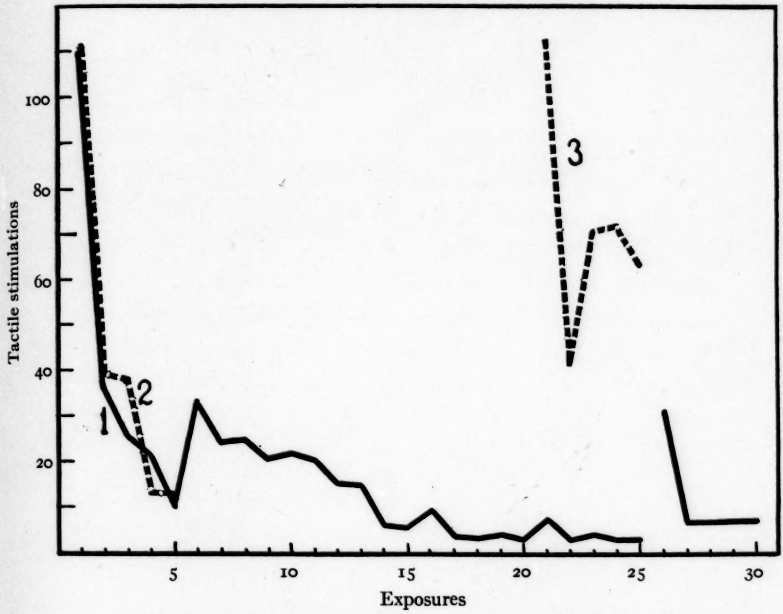

FIG. 1.—Every time a worm moved under light exposure, its snout was touched. Curve 1 represents the average number of tactile stimulations applied to seventeen worms during each 5-minute exposure to light separated by 30-minute intervals. The number of touches decreased with progressive exposures, indicating an acquirement of a conditioned inhibition to photokinesis. The rise at the twenty-sixth exposure is explained as having been due to a lapse of 10 hours between this exposure and the preceding one. Curve 2 represents the behavior of ten individuals with the tips of their snouts cut off when subjected to the same conditions. Curve 3 represents the behavior of twelve individuals which had not been stimulated tactually during the first twenty exposures, showing that light adaptation is not a factor in the modified response.

the initial one. Later on, some of the individuals would lie still without being once touched for one or more exposures in succession; others remained quiet as a result of receiving contact stimulation

only once or twice during an exposure. After several exposures some of the worms would respond as to a tactile stimulus even when the dish was lightly struck or when the surface film was broken, thus demonstrating heightened sensitivity to the stimulus in terms of the response, which phenomenon is concomitant with learning. During the course of the experiments *none* of the animals would remain entirely motionless without an occasional stimulation; a perfect stable modification of photokinesis was not produced. Although the inducement of immobility became easier in the case of every individual, the building-up of the modified response to light did not partake of the nature of a smooth progression but, rather, the worms would show occasional spurts of activity rendering individual curves very irregular and group curves somewhat so. Nevertheless, the general trend for every subject was for decreased movement under light exposure.

These results indicated that a conditioned response was produced in the flatworms, namely, an inhibition of the movements ordinarily induced by light. There were three other possible explanations, however: sensory adaptation to light might have taken place; motor fatigue might have set in; the inhibition might have been due to injury of the snout. To clear up these points, control experiments were carried out. In order to determine if sensory adaptation were a factor, twelve dishes each containing a fresh (untrained) individual were placed beside dishes containing experimental animals at the sixth exposure of the latter—the twentieth exposure of the controls thus falling in with the last exposure of the experimental animals. Hence, up to the twenty-first exposure to light the controls had not been touched. Beginning with the twenty-first exposure the controls were subjected to tactile stimulation in the same manner as the experimental animals; that is, every time they moved during the twenty-first exposure and the four exposures following, they were touched with the stick. Figure 1, curve 3, shows that photokinesis in them was of the same magnitude as it was in the experimental animals at the beginning. This eliminates the sensory adaptation factor. Also, the fact must not be overlooked that the time interval between exposures was 30 minutes. Holmes cites many examples of behavior modification through adaptation, in lower organisms, and

shows that its duration is only a matter of seconds. However, in the present experiment there was some retention of the modification after a 10-hour interval following the twenty-fifth exposure, which actually reveals an associative hysteresis comparable to memory.

During the first twenty exposures the controls were continually moving about in the dishes while the light was striking them. It is therefore reasonable to suppose that these individuals should have become as fatigued as did the experimental animals during the same number of exposures, especially since the latter became comparatively inactive before the twenty-first exposure. Contrary to what might be expected, the controls displayed no more signs of fatigue following twenty exposures to light than did the experimental animals at first—the control individuals in the twenty-first to the twenty-fifth exposures, inclusive, had to be touched just as frequently as did the experimental individuals in the first five tests (see Fig. 1, curve 3). The second factor, then, of motor fatigue is eliminated.

To find out if injury to the snout prevented the animals from creeping forward, a thin strip of tissue was cut off the anterior ends of ten worms. Two hours later these were exposed to light and stimulated in the same manner as the experimental animals were. Curve 2 of Figure 1 shows that the degree of their activity compared favorably with that of the normal worms during the first five exposures of the two groups. Therefore the inhibition in the experimental group was not caused by injury.

After eliminating the probability of sensory adaptation, motor fatigue, and injury as factors reducing the photokinetic creeping movements of the flatworm, I have shown the formation of a simple conditioned response in the polyclad, *Leptoplana*. Thus associative memory exists in organisms having the simple nervous organization of the phylum Platyhelminthes. The nature of the modification in behavior brought about in these experiments is an inhibition against the creeping movements normally induced by light stimulation. Simultaneous stimulation by light and prevention of forward motion has apparently been linked together in the form of a simple unstable association.

We cannot imply from these findings that the flatworm profits

by experience. In the first place, this behavior modification seems to be one of the simplest conceivable examples of associative memory or conditioned response. Notwithstanding its simplicity, many simultaneous presentations of the natural and conditioning stimuli are necessary before any appreciable facilitation can be brought about. Furthermore, the association is very unstable. Facilitation was built up in some individuals to such an extent that they would remain motionless during a whole exposure and less often during two or three in succession, but in no case did an individual lie still in more than five consecutive exposures. Occasional simultaneous presentation of the two stimuli was necessary throughout to maintain the negative response. If the experimenter failed to touch a "trained" worm after it had begun to move, it would usually creep as long as it was under the light; yet one touch might suffice to stop it. After only 10 hours had intervened between the twenty-fifth and twenty-sixth exposures, many touches with the stick were necessary to renew the association. The possibility that such inefficient functioning of associative memory is of advantage to an organism is doubtful, since the organism would adapt too slowly to changing environmental situations and would lose adaptations too rapidly to be of future service. It seems reasonable to suppose that the nervous system or cephalic ganglia of the flatworm have a function over and above that of mere transmission of impulses, namely, the retention of traces of experience, but this in such rudimentary form that in normal life it does not affect the animal's conduct. The organisms seem to have absolutely no ability to solve a problem: All the animals were conditioned against going forward, and in no case did a worm creep backward, even though it possessed a mechanism adequate to backward movement.

There is an interesting phenomenon in connection with the behavior of the light controls. When worms were stopped with a stick the first time they were exposed to light in the laboratory, the number of touches from exposure to exposure became progressively less, whereas the controls did not seem to be as amenable to training after being exposed twenty times without being touched. Figure 1, curve 3, reveals the fact that there was comparative little facilitation in the controls. The average number of touches for the first

five exposures of the fresh animals was 40.6, and that for the last five exposures of the light controls was 72.2. Furthermore, every one of the twelve controls began swimming sometime or other during nearly all of these last five exposures, whereas swimming occurred very infrequently in the experimental group. It is reasonable to suppose that light-movement became more strongly fixed in the controls, thus effecting an inhibition of an association between light and an opposite mode of response—immobility. The behavior of each of the twelve controls was consistent with the average result.

II. THE FUNCTION OF THE BRAIN

To determine if the "seat" of the conditioned response lay in the cephalic ganglia, these organs were removed from six specimens previously trained as described. After the thirtieth exposure, six dishes containing the animals were left on ice until the sea water approached 0°C. Then most of the tissue comprising the two ganglia in each of the anesthetized individuals was removed with a glass tube of small bore. Two hours' time was used to cool the animals, perform the section, and bring the water back to 18°. Then for five exposures they were subjected to the same treatment as had been given them previous to the extirpation of the ganglia.

After removal of the "brain" the animal moved forward by "ataxic" retrograde locomotion characterized by unrhythmical undulating muscular contractions, in contrast to the more rapid and efficient "ditaxic" retrograde locomotion of the normal animal, which is characterized by alternate projecting forward of one side of the body as if "walking" (Child, 1904). The slower motion was the result of removal of ganglia which are necessary to the ditaxic mode (Olmsted, 1922). In contrast to the normal, the anterior end took little part in locomotion. It behaved like a piece of sluggish or paralyzed tissue, folding occasionally. Since the snout is not projected in brainless worms, the number of touches with the stick per exposure has not exactly the same significance as before the operation. Nevertheless, the animal was stopped each time it was touched, not by a negative response of retracting, but simply because the stick held it back momentarily.

Five of the brainless worms began to move soon after the light

was turned on them, the sixth did not show movements of progression until the third exposure. Touching with the stick served merely as temporary impediments, any number of stimulations failed to stop them. Being more sluggish than normal ones, these animals would not start forward again as quickly after being touched. Consequently, fewer touches were administered during an exposure; the number of touches given per exposure might be taken as a rough

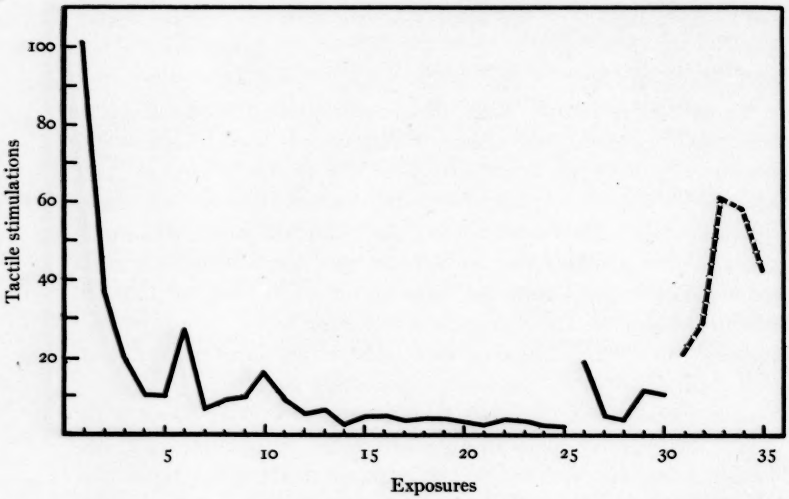

FIG. 2.—The solid line represents the average number of tactile stimulations per exposure for six worms. The dotted line represents their behavior after the cephalic ganglia were removed.

measure of the kinetic effect of the light. In spite of their sluggishness, these animals resisted the stick a greater number of times during all the five exposures than they did immediately before brain removal (see Fig. 2). In fact, the total number of touches during these five exposures of the six worms exceeded the total number given for the same six worms during the first five exposures in training: the averages per exposure per worm in the uncut condition was 35.2, and 41.6 in the operated condition.

The loss of the inhibition after brain removal might be accounted

for in any one of three ways. Cutting through the nerve tissue may have irritated the remaining nerve elements, but this is improbable inasmuch as the worms failed to move appreciably between exposures, and since controls, made by cutting chunks of tissue from the sides of four trained normal subjects, did not noticeably change their behavior. Secondly, the conditioned response may have involved only the anterior region which dominates the organism, so that, after cutting through nervous connections, the anterior ceased to exert control over the more posterior regions. The most reasonable explanation is found in the effect of removal of structures (the cephalic ganglia) which had been altered in some way as a result of the training. This fact suggests an analogy with the brain function of higher vertebrates, since the brain is the organ in which conditioning takes place (Pavlov, 1927).

Previous work on the cerebral ganglia of polyclad worms indicates that these ganglia have very special functions. Loeb (1902, chap. vi) showed that these ganglia are necessary to spontaneous creeping movements under ordinary conditions and that their removal decreases the speed of response. On the other hand, he also showed that the cerebral ganglia are unnecessary to the righting reaction. Olmsted (1922) showed that *Leptoplana* is unable to swim unless the whole brain is intact; that circus creeping movements to one side result from partial destruction of the ganglion on that side; that co-ordinate locomotion is not re-established when the brain is cut longitudinally. A. R. Moore (1923) showed that in the polyclad *Yungia* the brain is essential to both spontaneous creeping and swimming movements, for the reason that it lowers the threshold of stimulation. He found that the mechanisms for these movements are present in brainless specimens inasmuch as mechanical stimulation of the anterior median locus, chemical stimulation of the motor elements, or an increase of the ionic ratio $\frac{\text{Conc. Na+K}}{\text{Conc. Ca+Mg}}$ of the sea water elicits these movements in decerebrate individuals. Thus the brain is not necessarily a co-ordination center but rather acts as an amplifier of impulses. M. M. Moore (1928) has shown that the presence of the ganglia determines the orientation of *Leptoplana* under the influence of a constant electric current. My experiments indi-

cate that the ganglia have still another function—modifiability of response through individual experience.

The assumption is commonly made that a well-developed brain is necessary for associative memory. This view has been advanced chiefly by Loeb (1902, chap i). In order to classify the cephalic ganglia of a flatworm as a brain at all, the term must be stretched; and if they do constitute a brain, it certainly is not a well-developed one. These experiments indicate, then, that an elaborate brain is not necessary to associative memory; that a very simple one can possess this property to some extent. Thus experience leaves traces in the central nervous system although such traces are not of sufficient extent to be of practical consequence to the organism. It would seem, therefore, that the mechanism for associative hysteresis, as is the case with the other biological mechanisms, appears first as an experiment of nature, without immediate significance for the organism.

SUMMARY

Associative hysteresis has been demonstrated by the conditioned reflex method in the flatworm *Leptoplana*. The animal is photokinetic. Through simultaneously exposing the worm to light and preventing it from creeping forward, an inhibition of photokinesis was effected. Light was the biological stimulus for movement, and contact was the biological stimulus for prevention of movement. Light became also a conditioned stimulus for prevention of movement. Thus light was conditioned to inhibit a response which it itself elicited. Experiments with worms whose cephalic ganglia had been extirpated indicated that these organs took part in the learning.

The writer is indebted to Dr. A. R. Moore for suggestions and criticisms, to Mr. Milton Lindner for helping in the experiments, and to Dr. W. K. Fisher, director of the Hopkins Marine Station, for many courtesies extended to him during the course of the work.

LITERATURE CITED

BOHN, GEORGES. 1920. La naissance de l'intelligence (Paris: Ernest Flammarion).

CHILD, C. M. 1904. Studies in regulation. IV. Some experimental modifications of form regulation in *Leptoplana*. Jour. Exp. Zoöl., 95–133.

HOLMES, S. J. 1923. The evolution of animal intelligence (New York: Henry Holt & Co.).

JENNINGS, H. S. 1907. Behavior of the starfish *Asterias forreri de Loriol*. Univ. Calif. Pub. Zoöl., IV, No. 2, 53–185.

LOEB, JACQUES. 1902. Comparative physiology of the brain and comparative psychology (New York: G. P. Putnam's Sons).

MOORE, A. R. 1920. Stereotropism as a function of neuromuscular organization. Jour. Gen. Physiol., II, 319–24.

———. 1923. The function of the brain in locomotion of the polyclad worm, *Yungia aurantiaca*. *Ibid.*, VI, 73–76.

MOORE, M. M. 1928. Galvanotropism in marine polyclads, reported at Western Society of Naturalists, Claremont, California, June 15.

OLMSTED, J. N. D. 1922. The rôle of the nervous system in the locomotion of certain marine polyclads. Jour. Exp. Zoöl., XXXVI, 57–66.

PAVLOV, I. P. 1927. Conditioned reflexes (trans. by G. V. Anrep, Oxford Univ. Press).

YERKES, R. M. 1912. The intelligence of earthworms. Jour. Animal Behavior, II, 332–52.

STUDIES ON AMPHIBIAN METAMORPHOSIS. IV. GROWTH AND DIFFERENTIATION OF ANURAN TONGUE DURING METAMORPHOSIS

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INTRODUCTION

THE larval tongue of the anuran, as it occurs in tadpoles prior to the onset of metamorphosis, consists of a small, oval mass of undifferentiated tissue located on the posterior floor of the buccal cavity. Its free surface is slightly convex in shape, while the entire mass is firmly imbedded and attached to the lining epithelium of the mouth. At this time it is deep pink in color; but later, when stimulated to growth during the early stage of metamorphosis, it becomes quite reddish in appearance, the result of increased vascularization.

The growth and differentiation of this larval structure into the highly specialized, bifid tongue of the adult frog presents one of the most remarkable transformations of anuran metamorphosis. Shortly following the first noticeable external signs of metamorphosis, the oval mass of cells undergoes rapid growth and soon tends to assume an elongated shape. At the stage of metamorphosis when the fore limbs are normally released from the opercular cavity, the tongue is approximately 4 mm. in length and tapers from about 2 mm. in width at its posterior end to about 1 mm. in width at the anterior end. It is still, however, firmly attached at all points to the floor of the mouth. Following this stage, a period of rapid growth ensues, which may be as great as 1 mm. in length a day. The posterior border of the mass shortly bifurcates to develop into the two cornua of the adult structure and soon becomes detached from the hyoid cartilage beneath. Further growth and differentiation result in the development of the various internal muscular tracts and a highly specialized covering of mucous membrane containing filiform and fungiform papillae and numerous mucous glands.

The principal problem with which the present paper is concerned

has to do mainly with the probable factors responsible for the rapid growth and differentiation of the larval tongue within the buccal cavity. Previous papers of the writer have brought out the conclusion that certain metamorphic changes of involuting larvae are not the direct result, at least, of the thyroid hormone operating through the blood stream. In other words, although the activity of the thyroid gland as the causal agent of anuran metamorphosis has been clearly demonstrated by Gudernatsch (1912, 1914), Allen (1919), Swingle (1918, 1923), *et al.*, the exact mechanism by which its hormone or hormones initiate the numerous differentiations, disintegrations and dedifferentiations of structures, during metamorphosis, is little known. In many cases it seems quite probable that a complicated interlocking series of factors intervene between the primary activating influence of the thyroid gland and the various end-results. The writer has shown, for example (1926), that the histolysis of opercular integument during the formation of the opercular, fore-limb perforations is not due to the direct action of a hormone carried by the blood stream but is the result of histolytic influences transmitted to the opercular integument by contact with the atrophying gills. Similarly, the writer (1928) has shown that the dedifferentiation of integument to form the integumentary portion of the tympanic membrane is due to contact with the developing annular tympanic cartilage. The possibility, therefore, of adjacent structures of the buccal cavity influencing the growth and differentiation of the larval tongue was apparent and thought worthy of investigation.

Obviously, extirpation and transplantation of the larval tongue before differentiation or marked growth had begun would throw light on the problem. Should such transplantation prove feasible and normal growth and differentiation subsequently develop during the course of metamorphosis, the conclusion could be drawn that adjacent structures of the buccal cavity are not concerned but, instead, direct hormonic factors operating through the blood stream. Conversely, failure of such transplants to undergo normal growth and differentiation during metamorphosis would indicate the reverse hypothesis of one or more necessary intervening factors. Operations were consequently designed to clarify the foregoing questions.

MATERIALS AND METHODS

Fully grown larvae of *Rana pipiens* were employed for all operations. The larvae were collected during the month of July from a small pond in the vicinity of Iowa City, Iowa. The stock, when brought to the laboratory, was given generous feedings of *Spirogyra* for several days prior to selection of the healthiest individuals for operative work. Those selected measured from 75 to 95 mm. in length and were either on the verge or in early stages of metamorphosis. The undifferentiated larval tongue in such individuaia was usually still oval in shape, although in a few, slight elongation had occurred. The operations were carried out under anesthesia, a 0.05 per cent aqueous solution of chloretone being used for this purpose.

The technique of extirpation productive of the best results was briefly as follows: The anesthetized tadpole was first placed in a specially constructed paraffin holder. This latter consisted of two blocks of paraffin so shaped that the anesthetized larva could he held firmly, yet safely, between them in a vertical position with only the head exposed. The paraffin holder with the inclosed tadpole was then placed on the stage of a dissecting microscope, pressure applied at the sides of the jaws to open the mouth, and a strong beam of light focused into the buccal cavity to illuminate the anlage to be extirpated. For the actual extirpation, a fine-pointed pair of thumb-spring scissors was found to be adequate. The mucous membrane surrounding the oval mass was carefully cut, and the anlage finally removed intact and placed in a watch crystal of Ringer's solution. The larva was then removed from the holder, placed upon a damp cloth, and a triangular flap of back skin laid back for the reception of the transplant. In a few cases the transplants were simply placed upon the musculature of the back. In the majority of cases, however, a small incision was first made in the back muscle and the transplant inserted partially in the cut. The flap of integument was then replaced, and the animal placed in shallow water to allow exposure of the wounded area to the air. After a half-hour of such exposure, the flap of integument had adhered sufficiently to allow total immersion of the animal in water, where recovery from the anesthetic shortly followed. Subsequent treatment involved daily changes of the aquaria water and the application of compressed air

to insure a sufficient supply of dissolved oxygen. The fact that the animals were usually undergoing early metamorphosis made feeding unnecessary.

MACROSCOPIC APPEARANCE OF GRAFTS

Thirty-six successful transplantations were made in all. The rate of transplant growth was subject to considerable variation, however. In general, growth was immediate and sufficient to produce a distinct bulging of the integument of the back, covering the transplant, within 5–7 days. At the end of this period, however, the larvae were rapidly completing the later stages of metamorphosis, and the mortality rate consequently became high.

Eight individuals survived complete metamorphosis, the latter condition being reached approximately 2 weeks following the transplantation period. Several of these were killed at this time, the transplants examined, and then fixed for later histological examination. The remaining individuals lived from 4 to 8 days longer and, upon dying, were likewise examined and preserved. Macroscopic examination of the transplants, *in situ*, revealed oval or slightly elongated masses of tissue of various dimensions. The largest of these measured 4.5 mm. in length, 3.5 mm. in width, and 2 mm. in thickness and represented an enlargement of the original transplant of 1,500–2,000 per cent. In general, however, the volume of the transplants had increased about 1,000 per cent. With but one exception, they were firmly attached to the musculature of the back and appeared to be fairly well vascularized. Fine blood vessels were noticeable in a few cases, ramifying near the surface of the transplants. It must be remembered, however, in this regard that the transplants undoubtedly derived much of their nutrition from the lymph in which they were continually bathed, the subcutaneous lymph spaces of the frog serving as admirable culture chambers.

In general the transplants were characterized by the absence of any signs of bifurcation at their posterior borders. Slight evidences of cornua formations were observed in several cases, however. Subsequent examinations of the buccal cavities of all individuals revealed that extirpation had been completed in all but three of the cases. Small masses of tissues had regenerated in these instances

which presented no signs of bifurcation. Distinct movement or twitching of the intact transplants were observable in many cases, giving evidence of muscular development and probably of some degree of innervation. Histological examinations later confirmed the presence of muscle fibers.

MICROSCOPIC APPEARANCE OF TRANSPLANTS

Examination of sections made of the more developed transplants gave evidence that considerable differentiation had occurred. The bulk of the internal tissue mass consisted of loose connective tissue in which could be found occasional bundles of striated muscle fibers. The latter, however, were never grouped into formations typical of the genioglossus and hyoglossus muscle fibers of the normal tongue. In fact, the muscle fibers present appeared to be quite uniformly distributed throughout the transplant.

The most-pronounced differentiation was found in the presence of a well-developed mucous membrane. Numerous mucous glands were present of a more or less uniform size and distribution. Definite filiform and fungiform papillae were likewise developed. The former were, in general, less numerous and smaller than is true of the normal condition. In general, they also appeared to be less slender and somewhat shorter as compared with those of the normal organ. The fungiform papillae were likewise less numerous and smaller than normal papillae of this type and were found less frequently than filiform papillae. In length they were somewhat shortened as compared to the normal condition, and their distal ends were generally less typically expanded. In general it might be said that differentiation of the mucous membrane and its composite elements was considerably advanced, requiring only additional time for complete development.

DISCUSSION

The results of the larval tongue transplantations clearly indicate that the factors necessary for the growth of this structure during metamorphosis are adequately supplied wherever nutrition is obtainable. It would seem in this regard that nutrition and early growth are possible in a medium of lymph, although later development is more adequately and quickly reached where the normal

vascular system is operative. The fact that the transplants never attained a growth comparable to that of normally developing tongues of the buccal cavity was probably due to the retardation of such vascularization and also to the abnormal general environment encountered. Of the latter, pressure of the surrounding integument of the back and lack of exposure to air might well have retarded growth, in that these conditions are never encountered within the buccal cavity.

The failure of the transplants to develop the normally bifurcated shape of the adult tongue is difficult of interpretation. It will be recalled, however, that normal development within the buccal cavity involves several factors not found in transplanted tongues. Normal development is obviously chiefly restricted to lateral and dorsal expansion due to the flat, unyielding surface of the hyoid forming the posterior ventral floor of the mouth and upon which the posterior regions of the developing tongue rest. The ingrowth of the hyoglossus muscle from either side may also be a determining factor in the shaping of the organ. In the transplants, growth was unrestricted at all points, which probably accounted for the tendency to form more-or-less regular, ovoidal masses. The insertion of the posterior portion of the transplants into the musculature of the back may also have operated to hinder the normal development of the cornua which normally form following bifurcation of this region. It seems quite probable, however, that had sufficient time been available for development, cornua formation would have resulted. The slight signs of bifurcation noticed in several cases would seem to justify this assumption.

The differentiation of a complete mucous membrane containing filiform and fungiform papillae and numerous mucous glands indicates quite clearly that the development of this tissue is not dependent on formation within the buccal cavity. The sole requisite for its development and that of its component elements would appear to be an adequate vascular supply and sufficient time. In this regard lack of time was no doubt responsible for the somewhat abnormal shapes of the papillae, which resembled more the developing stages than the complete structures of normal papillae.

The growth and differentiation of the larval tongue of the anu-

ran, during metamorphosis, would seem to be chiefly due, therefore, to hormonic influences liberated through the blood supply at an early stage of larval transformation. Whether this influence consists simply of the thyroid hormone itself or of a secondary secretion liberated by the activity of the former is quite problematic. The idea that the thyroid hormone is not directly but only initially responsible is favored by the fact that growth and differentiation of the larval tongue do not begin until the larva has arrived at a certain stage of metamorphosis. The results further indicate that adjacent structures of the buccal cavity have no primary influence on growth or differentiation but may have a secondary rôle in determining the shape of the adult structure.

SUMMARY AND CONCLUSIONS

1. Autoplastic transplantation of larval tongues to the back of metamorphosing *Rana pipiens* larvae was successful in over 90 per cent of the cases.

2. The transplants generally developed into oval-shaped masses of tissue containing scattered bundles of muscle fibers, loose connective tissue, and blood vessels. In general the external shape of the normal tongue was not attained, although slight indications of bifurcation occurred in several cases.

3. A well-developed mucous membrane was formed containing filiform and fungiform papillae and numerous mucous glands.

4. The conclusion is drawn that growth and differentiation of the anuran tongue is the result of hormonic influences within the blood stream which are liberated or activated at a specific stage of metamorphosis. Adjacent structures of the buccal cavity have little or no influence except as they may possibly function in determining the external shape of the adult structure.

LITERATURE CITED

ALLEN, B. M. 1919. The relation of the pituitary and thyroid glands of *Bufo* and *Rana* to iodine and metamorphosis. Biol. Bull., No. 6.

GUDERNATSCH, J. F. 1912. Feeding experiments on tadpoles. I. The influence of specific organs given as food, on growth and differentiation. Arch. f. Entwick.-mech., Bd. XXXV.

———. 1914. Feeding experiments on tadpoles. II. A further contribution to the knowledge of organs of internal secretion. Amer. Jour. Anat., Vol. XV.

HELFF, O. M. 1926. Studies on amphibian metamorphosis. I. Formation of the opercular leg perforation in anuran larvae during metamorphosis. Jour. Exp. Zoöl., Vol. XLV.

———. 1928. Studies on amphibian metamorphosis. III. The influence of the annular tympanic cartilage on the formation of the tympanic membrane. Physiol. Zoöl., Vol. I, No. 4.

SWINGLE, W. W. 1918. The acceleration of metamorphosis in frog larvae by thyroid feeding, and the effect upon the alimentary tract and sex glands. Jour. Exp. Zoöl., Vol. XXIV, No. 3.

———. 1923. Thyroid transplantation and anuran metamorphosis. *Ibid.*, Vol. XXXVII, No. 2.

LATERAL GRAFTS AND INCISIONS AS ORGANIZERS IN THE HYDROID, CORYMORPHA

(Twenty-five figures)

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IN A recent paper (Child, 1927*b*) it was shown that a simple transverse lateral incision in the middle or proximal naked stem region of *Corymorpha* heals without outgrowth but that an incision with lacerated margins at the same stem levels may in some cases determine the development of a new hydranth and stem and under certain conditions a complete axis. In these experiments incisions were made only in the middle and proximal regions of the stem, the basal end and the original hydranth were removed, and the developing terminal hydranth of the original axis was cut off every 2 or 3 days as a new one developed. During the summer of 1928 a study of the fate of small pieces of the naked stem region implanted in lateral incisions in the stem was begun. In the summer months the *Corymorpha* material always shows more or less deterioration and decrease in size, but during the autumn it recovers. Breeding goes on, however, during the summer, and the larger individuals of autumn and winter are of course later generations than the summer individuals. In 1928 the animals finally became so small and were in such poor condition that the experiments were discontinued, but the experiments performed demonstrated that even a very small piece of the naked stem implanted in a lateral incision may determine a new axis, of which it constitutes only a very small part, the remainder developing from the host tissues. In other words, the graft may act as an organizer of the host tissues or, more strictly speaking, as a reorganizer. Moreover, it was also found that under certain conditions even a simple incision may organize a new axis. Some data on the relation of level from which the graft is taken and level of implantation to the result have been obtained, but many other aspects of the problem remain for future investigation. Nevertheless, the data already at hand establish beyond question the

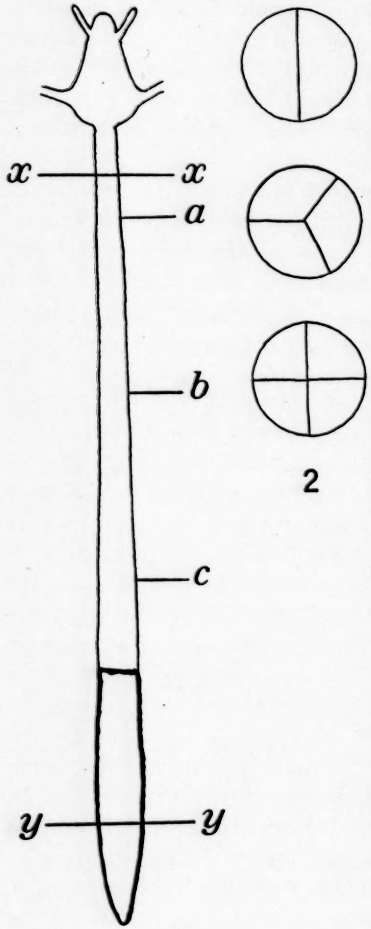
organizing action of the graft, and the chief purpose of the present paper is to record the evidence on this point. It should be noted, however, that this is not the first case of a graft acting as organizer among the Hydrozoa. Twenty years ago it was shown by Browne (1909) that in *Hydra* a small graft from the region at the base of the tentacles was able to organize a new axis, in large part from the host tissue.

Once more, I desire to acknowledge my indebtedness to Dr. T. Wayland Vaughan, director of the Scripps Institution of Oceanography at La Jolla, California, for making this work possible by placing at my disposal the facilities of the station.

METHOD

In the experiments to be described only very small pieces of the stem have been used, so small that if isolated they would give rise only to the extreme apical regions of hydranths (Child, 1926*b*, pp. 772–75). Pieces 1–2 mm. in length cut from the desired level of the naked stem region (Fig. 1) by transverse planes of section were used entire in a few experiments, but in most cases the pieces were cut into sectors, halves, thirds, or fourths (Fig. 2), and one such sector was implanted. In cutting these very small pieces, the cells of the entodermal parenchyma which forms the supporting core of the stem are usually in large part crushed or at least collapsed. Such injury, or even the complete loss of the entodermal parenchyma, does not essentially affect reconstitution, for the ectoderm and the entoderm of the longitudinal canals are the tissues chiefly concerned in this process; and in the more extreme degrees of reorganization the entodermal parenchyma is in large part or wholly lost. As soon as possible after these small pieces are prepared, the hydranth and the basal tip with the mud clinging to the holdfasts are removed from the host stem, which then represents the region between *xx* and *yy*, Figure 1, and a lateral transverse incision is made in the stem with small scissors at the level at which the piece is to be implanted. This incision is made as small as will permit the insertion of the graft. In the experiments to be described it involves approximately one-third of the circumference of the stem, severing the ectoderm and the entodermal canals and cutting more

or less deeply into the entodermal parenchyma. A needle is then inserted into the open incision and its point moved about in the entodermal parenchyma in order to crush some of the cells and so make room for the insertion of the graft. Then with the aid of needles the small piece is inserted in the incision without any attempt at orientation.



FIGS. 1 and 2.—Diagrammatic outlines indicating levels of origin and implantation of grafts (Fig. 1) and character of pieces grafted (Fig. 2).

Unless held in place for a short time, the graft is usually forced out again in the course of a few seconds by contraction of the stem, but, by keeping the stem bent with the incision on the concave side for 1–2 minutes, retention of the graft is practically assured. The bending of the stem approximates the edges of the incision for the time being and holds the graft in position, and within a minute or two it adheres to the host tissue sufficiently to remain in position, with rare exceptions, e.g., after rough handling producing strong contraction. Union between graft and host takes place more readily when the cut surfaces are freshly made, but it has not yet been determined how long a cut surface remains capable of union. The small pieces used for grafts contract strongly if they remain isolated, and within an hour or

two become rounded so that little or no cut surface remains exposed.

The grafts were usually made in a large watch glass or a tissue-culture depression slide, and immediately after insertion of the graft the stem was bent with the incision on the concave side and held in that position for the necessary length of time. This was most readily accomplished by sliding the stem out of the water in the bent position. On the glass with only such water as adhered to the stem it was much more easily kept in the bent position. After at most 2 or 3 minutes, the stem was returned to water with as little disturbance as possible, and the Stender containing each lot of five or ten stems was placed in a large aquarium in flowing water. In all experiments performed thus far, the host stem retained a part of the basal region with holdfast buds; and within a few hours new holdfasts grew out from these buds and attached (Child, 1928*a*) and the stem became erect. The erect position insures that development of the graft shall not be inhibited by contact with the substratum and that conditions for the grafts at corresponding levels of different stems shall be as nearly uniform as possible.

For controls, stems of the same length as the hosts were taken, their hydranths and basal tips removed as in the hosts, and a lateral incision made as nearly as possible at the same level of the stem as in the hosts. Into the open incision a needle was inserted and moved about in the entodermal parenchyma so as to crush some of the cells as in the hosts. These controls were kept under the same conditions as the grafted stems.

In cases in which the graft determines a new axis, the rate of development of the hydranth varies according to conditions, as will appear below. In the most rapidly developing cases the hydranth is often well formed and the tentacles 2–3 mm. in length, and in some cases a stem proximal to the hydranth is already present within 48 hours after grafting.

The figures showing results of grafts and incisions were drawn from sketches of living individuals. In certain respects they are diagrammatic, that is, the number of tentacles shown is only approximate, except where otherwise noted, and the developing gonophores and holdfasts are not shown. As regards the position of the

new axis, its stage of development and size as compared with the terminal hydranth and the portion of the new axis formed by the graft itself, the figures represent as nearly as possible in each case the actual conditions observed. Figures 16–25 are drawn on a somewhat larger scale than Figures 3–15.

THE OCCURRENCE OF NEW AXES IN RELATION TO GRAFTS AND INCISIONS

Data concerning grafts in the proximal naked stem region of small pieces from approximately the same level and of pieces from a

TABLE I

FREQUENCY OF DEVELOPMENT OF NEW AXES FROM GRAFTS OF PROXIMAL AND OF DISTAL PIECES AT A PROXIMAL LEVEL OF THE STEM. CONTROLS ARE STEMS WITH INCISION AT THE PROXIMAL LEVEL BUT NO GRAFT

	Series	Length of Host in Mm.	Length of Donor in Mm.	No. of Stems	Character of Piece Grafted	New Axis	No Outgrowth	Dying
Pieces from levels 5 mm. distal to perisarc implanted at same level	28	40–50	25–30	10	¼ sector	6	3	1
	33	40–50	40–50	5	¼ sector	1	4	.....
	39	30	30	5	¼ sector	2	3	.....
	Total number of new axes, 9 = 45%							
Pieces from levels 2–5 mm. proximal to hydranth implanted at level 5 mm. distal to perisarc	15	50–70	25–30	5	whole	5	.....	.....
	16	50–70	20–30	5	½–⅓ sector	4	1	.....
	21	60	60	5	whole	4	.....	1
	22	60	20–25	5	½ sector	2	3	.....
	34	30–50	30–50	10	½–¼ sector	10	.....	.....
	Total number of new axes, 25 = 83.3%							
Controls; lateral incisions 5 mm. distal to perisarc with injury to parenchyma	17	50–70	.....	10	.....	.....	10	.....
	35	30–40	.....	10	.....	.....	10	.....
	Total number without new axes, 20 = 100%							

distal level, together with controls, are given in Table I. The proximal level from which proximal grafts were taken and at which they were implanted was approximately 5 mm. distal to the distal end of

the perisarc (Fig. 1*c*), or somewhat less in the shorter animals. The levels from which distal grafts were taken were 2–5 mm. proximal to the base of the hydranth (between *xx* and *a*, Fig. 1). The various columns of Table I require no extended explanation, but attention may be called to certain points. First, as the columns headed "Length of Host" and "Length of Donor" indicate, donor and host are approximately the same length in some series, but in other series the donor is smaller and physiologically younger than the host. In the first section of the table, giving grafts of proximal pieces at a proximal level, the donors are somewhat smaller than the hosts in the first series (Ser. 28) and the same size in the other two (Ser. 33, 39). In the second section, giving grafts of distal pieces at a proximal level, the donors are smaller than the hosts in the first three series (Ser. 15, 16, 22) and the same size in the other two (Ser. 21, 34). The controls include one series of the largest individuals available and one of smaller individuals. Second, the grafts were in all cases either transverse segments of the stem cut as short as possible (1–2 mm.), but including the whole circumference of the stem, or they were sectors representing one-half, one-third, or one-fourth of such pieces (Fig. 2). In the column "Character of Piece Grafted," these differences are indicated by "whole," "$\frac{1}{2}$ sector," etc. In the first section of Table I, proximal pieces grafted at a proximal level, the pieces are all one-fourth sectors, but this does not mean that they are smaller than the distal pieces of the second section, which range from whole segments to one-fourth sectors. The diameter of the stem is so much greater at the proximal level that the proximal pieces grafted are fully as large as, and often larger than, the distal pieces. Within the range used in these experiments size of piece apparently has little or no effect on the ability of the graft to organize a new hydranth, but it is probable that with a wider range of size such an effect may appear.

The first section of Table I, grafts at a proximal level of pieces from the same level, shows nine out of twenty cases, 45 per cent, developing new axes; ten, 50 per cent, remaining without new axes and one dead. The second section, grafts at a proximal level of pieces from a distal level, shows development of a new axis in 83.3 per cent. The third section, controls, consisting of lateral incisions at the

proximal level without implants shows no development of new axes from the incisions. The data demonstrate beyond question that the new axes are really determined by the grafts and not by the incisions alone. They also indicate that distal pieces grafted into a proximal level are more effective in determining new axes than proximal pieces grafted at the same level. While this apparent difference is in accord with the gradient conception, since the distal piece represents a higher level of the gradient than the proximal, a larger number of cases is desirable as a basis for percentage differences. The data of Table I are sufficient to establish beyond question the point of chief importance, viz., that grafts of small stem pieces may determine new axes.

The results with grafts at distal levels of the stem are less striking than those with grafts at proximal levels, because at distal levels the incision itself, without a graft, may determine a new axis. The data obtained at distal levels are given in Table II. They include only grafts from proximal levels and control grafts, grafts from distal levels at distal levels not having been made. In consequence of the much greater contractility and change in length of the more distal regions of the stem, the variation in level of the incisions (approximately from *a* to *b*, Fig. 1) is greater than in the proximal region. Incidentally, these grafts and incisions at distal levels give information concerning the length of stem involved in the reconstitution of a new hydranth. In individuals 50 mm. or more in length grafts implanted 10–15 mm. from the distal cut end are often included in the new hydranth region and give rise only to a duplication of the more distal levels of the hydranth (see pp. 356–60). This means that a length of 10–15 mm. of the stem is involved in the development of a hydranth which is at first only 3–4 mm. in length.

The column of Table II headed "New Axis" is subdivided into two parts to separate the cases in which the new axis determined by the graft or the incision is entirely proximal to and separate from the terminal hydranth region from those in which it is within the hydranth region and constitutes a partial duplication of the hydranth. Otherwise Table II is similar to Table I. The first section of Table II, giving the data on grafts of proximal pieces at distal levels, shows development of new axes proximal to the terminal

hydranth in 37 per cent of the cases and new axes constituting a partial duplication of the hydranth in 53 per cent, a total of 90 per cent. The controls, however, with incision but no graft at the distal level, show 33 per cent of new axes, 23 per cent proximal to the terminal hydranth and 10 per cent in the hydranth region. Evidently at these stem levels the incision alone is often sufficient to determine a new axis. This is in marked contrast to the proximal

TABLE II

FREQUENCY OF DEVELOPMENT OF NEW AXES FROM GRAFTS TAKEN FROM A PROXIMAL LEVEL AND IMPLANTED AT A DISTAL LEVEL. CONTROLS ARE STEMS WITH INCISION AT THE DISTAL LEVEL, BUT NO GRAFT

	Series	Length of Host in Mm.	Length of Donor in Mm.	No. of Stems	Character of Piece Grafted	New Axis: Proximal to Terminal Hydranth	New Axis: In Terminal Hydranth Region	No Outgrowth	Dying
Pieces from levels 5 mm. distal to perisarc implanted at levels 5–15 mm. proximal to hydranth	18	50–70	20–25	5	whole	3	2	.....	.....
	19	50–70	20–25	5	½–¼ sector	.....	5	.....	.....
	25	50–60	50–60	5	½–¼ sector	1	4	.....	.....
	29	40–50	40–50	5	¼ sector	2	.....	3	.....
	31	40–50	30–40	5	¼ sector	5	.....	.....	.....
	32	30–40	40–50	5	¼ sector	.....	5	.....	.....
	Total number of new axes, 27 = 90%								
Controls; lateral incisions 5–15 mm. proximal to hydranth with injury to parenchyma	20	50–70	.....	10	.....	3	3	2	2
	30	40–50	.....	10	.....	1	.....	7	2
	36	30–50	.....	10	.....	3	.....	7	.....
	Total number of new axes, 10 = 33.3%								

levels, at which no case of the sort has been observed thus far (Table I) unless the margins of the incision were lacerated (Child, 1927*b*). This difference in the effect of the incision at different levels is undoubtedly an expression of the difference in physiological condition at distal and proximal levels of the gradient. In some cases the cells of the distal level are sufficiently activated by the incision alone to initiate development of a new axis, while at the proximal level the incision must either be lacerated or a graft inserted to determine a

new axis. But even at the distal levels the grafted stems show a frequency of new axes almost 60 per cent higher than those with incisions only. Evidently the reorganizing graft is more effective as an organizer than the cells activated by the incision. This difference may be due to more intense activity or to greater number of active cells in the case of the graft.

A comparison of the frequencies of new axes from proximal pieces grafted at proximal levels (Table I) and proximal pieces grafted at distal levels (Table II) seems to show that the latter are somewhat more effective as organizers, since they show almost 60 per cent more new axes than the controls while the proximal pieces at proximal levels show only 45 per cent. This difference, however, cannot be regarded as significant, in view of the small number of experiments and also because, as will appear below, the graft at the distal levels apparently does not always act as organizer when it is present (pp. 356–57). Apparently the distal pieces grafted at a proximal level are somewhat more effective as organizers (83.3 per cent) than the proximal pieces at distal levels (57 per cent above the controls), but further data are desirable on these points.

Tables I and II give the data obtained thus far, except for some preliminary experiments which were primarily concerned with technique. These were in most cases single experiments with grafts from various levels implanted at various levels, but as far as they go they are in full agreement with the tabulated data. The experiments were discontinued in the latter part of August on account of the small size and poor condition of the material, but they proceeded far enough to demonstrate beyond question the effectiveness of small grafts and, at certain levels, of incisions as organizers of new axes and to indicate some of the differences in effectiveness at different levels of the stem. More extended experiments with the much larger individuals of late autumn and winter may provide more definite data as regards effect of size of graft and of host and donor, level of graft and level of implantation, and various other factors.

THE DEVELOPMENT OF THE NEW AXES

In the course of a few hours after implantation the graft undergoes considerable contraction, becomes rounded, more or less opaque

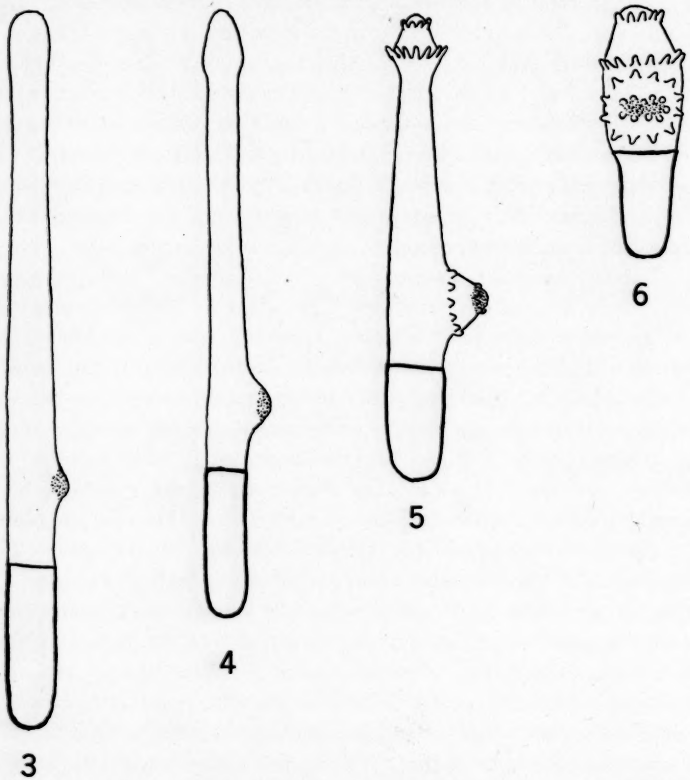
and sooner or later usually appears more or less pinkish in color as contrasted with the more translucent tissue of the host stem. At first the graft lies in the open incision, often protruding slightly; but the incision closes rapidly in most cases and within 12–15 hours is no longer visible, and the graft is incorporated into the stem tissue but is still clearly visible and has a definite boundary. In case of resorption without outgrowth, the graft may remain distinct for several days as a more or less opaque region of the stem in which the longitudinal canals are lacking, usually undergoing more or less elongation during this period. Gradually the graft area becomes like the rest of the stem in general appearance, though irregularities of the entodermal canals may persist in it throughout the laboratory life of the animal concerned.

The development of grafts at proximal levels.—Figure 3 shows an early stage; Figure 4, a somewhat later stage of development of the new axis from a graft. In Figure 4 the outgrowth of the host tissue is beginning, the graft forming its apex; and in Figure 5 the form of the hydranth is appearing and tentacles are already present. In these and following figures the graft is indicated by shading; and it will be seen that in Figure 5 the graft forms only the hypostome and perhaps the distal tentacles, the remainder of the hydranth body developing from the host tissue. In later stages this case completed the hydranth body and developed a stem from the host tissue like other new axes at this level. In Figure 6 an early hydranth stage is shown in which the graft gives rise to two hypostomes close together but determines only a single hydranth structure from the host tissue. In this case the stem underwent marked shortening, so that practically the whole preperisarcal region became involved in the development of the terminal and the graft hydranth. Such shortening is common in stems in poor condition and is often followed by death.

As the lateral hydranth is determined by the graft, the reorganization of the lateral stem region of the host becomes clearly visible in the living animal, even before the proximal tentacles develop. In the region undergoing reorganization, approximately a circular area about the graft, the longitudinal entodermal canals disappear, the tissue becomes more translucent, and a little later the

primordia of the proximal tentacles appear as localized growth regions about the periphery of the circle.

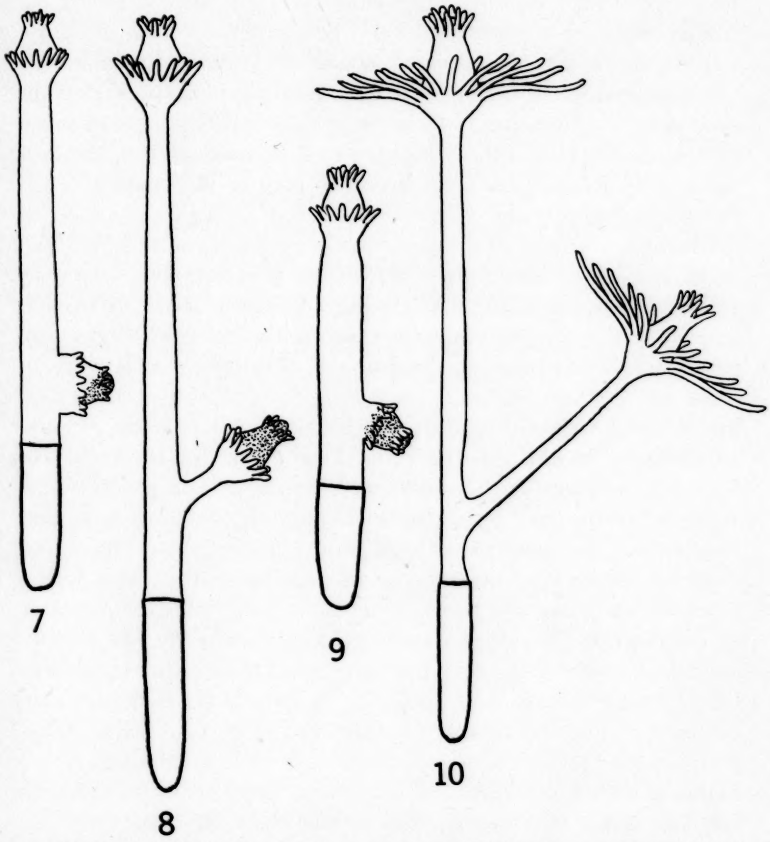
Figures 7, 8, and 9 show more advanced stages about 48 hours after grafting. In Figure 7 the graft is oblique, giving rise to the



FIGS. 3–6.—Eighteen- to 48-hour stages of development of new axes from distal pieces grafted at a proximal level, the graft being indicated by shading. Figures 3 and 4, stages preceding hydranth formation. Figures 5 and 6, early hydranths; Figure 6, with two hypostomes developed from the graft.

hypostome and one side of the manubrium. In Figure 8 it gives rise to the whole manubrium, and in Figure 9 to the whole hydranth. All gradations between cases like Figures 5 and 6, in which the graft

forms only the hypostome, and those like Figure 9, in which the whole hydranth body develops from it, have been found; but in no case thus far observed has the graft taken any part in the formation of the new stem.



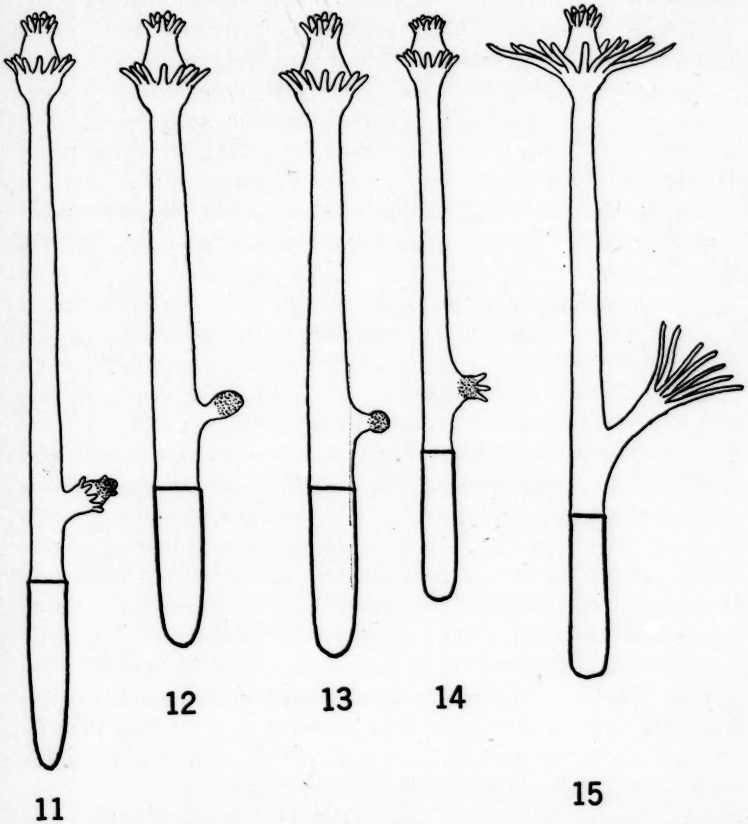
FIGS. 7–10.—Later stages of new axes from distal grafts at a proximal level. Figures 7–9, 48-hour stages, indicating by shading the portion of the hydranth formed by the graft. Figure 10, an advanced stage, showing development of stem.

All hydranths from grafts at proximal levels develop a stem (Figs. 8, 10) which usually attains considerable length, as in Figure

10, and may become equal in length to that part of the host axis distal to the level of the graft. Frequently, also, the lateral hydranth organized by the graft becomes more or less dominant over the host hydranth, and the host axis distal to the level of the graft axis undergoes shortening. In some cases observed, the host hydranth has undergone complete regression and resorption, and the graft hydranth has become the terminal hydranth. It seems probable that the graft hydranth, arising, as it does, from the lateral region of the host stem and from the graft, involves a more extensive reorganization than the hydranth developing at the distal end of the host stem; consequently, the graft hydranth may be the younger of the two physiologically and dominate because of its higher metabolism.

Figures 3–10 are all cases of distal pieces grafted at a proximal level. Proximal pieces grafted at the same proximal level determine hydranths less frequently than the distal pieces, as shown in Table I; and when they do determine hydranths, the determination is apparently less rapid than in the grafts of distal pieces at a proximal level. In the case of distal grafts at proximal levels, the lateral hydranths determined by the grafts usually develop almost or quite as rapidly as the terminal hydranths (Figs. 5–10). But the hydranths from proximal grafts at a proximal level develop in general more slowly than the host hydranth, and the graft hydranth is at first smaller than the host hydranth. Figure 11 shows the most advanced stage of development observed 48 hours after grafting in any case of a proximal graft at a proximal level. In most cases the new axes at this time are in earlier stages, often approximately like Figures 12 and 13, in which the graft has determined more or less outgrowth but no tentacles have developed and the form of the hydranth may be evident (Fig. 12) or not yet developed (Fig. 13). In the distal grafts at the proximal level, development is often as far advanced as Figure 9 within 48 hours, and only rarely have tentacles failed to develop within this period. Among the observed cases, approximately 90 per cent of the hydranths developing from distal grafts at proximal levels show tentacles within 48 hours, while only 55 per cent of those from proximal grafts at the same level attain a tentaculate stage within 48 hours. Here again, however, larger numbers are desirable. In one case of a proximal graft at a proximal

level the graft apparently began to develop as if it were an isolated piece, and gave rise to an apical region with three distal tentacles, a form very frequent in such small pieces (Child, 1926*b*), before the



FIGS. 11–15.—New axes from proximal grafts at a proximal level. Figures 11–14, 48-hour stages. Figure 15, an axis with nothing distal to the proximal tentacles.

rest of the hydranth had even attained definite form (Fig. 14). It is probable that in this case the union between graft and host was not complete in the early stages, so that the graft began its development more or less independently. This case later developed a complete

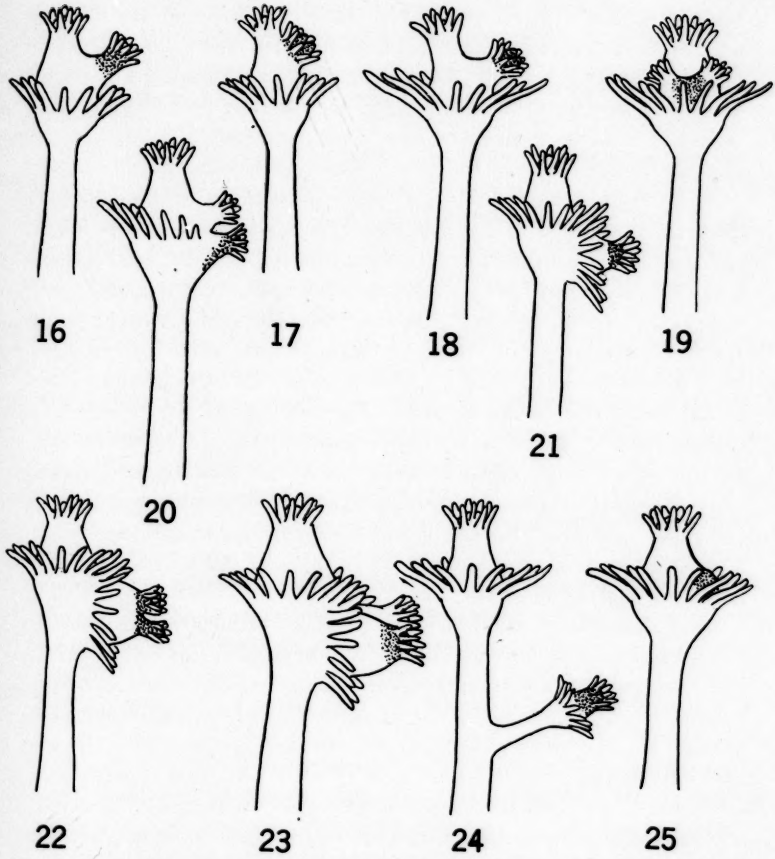
hydranth and stem. In two cases the proximal graft at the proximal level developed only a proximal hydranth region with a few proximal but no distal tentacles (Fig. 15). In such cases the graft is apparently more or less inhibited by the host or is itself not sufficiently active to give rise to the more distal levels of the hydranth. Thus far the distal grafts at the proximal level have shown no such cases.

It appears evident from these facts, even though the number of cases is not large, that the proximal grafts at a proximal level are in general less effective as organizers than the distal grafts at the proximal levels. The proximal grafts not only determine new axes less frequently than the distal, but the new axes determined develop less rapidly and the hydranths are smaller and occasionally lack the distal regions.

The development of grafts at distal levels.—As already noted in connection with Table II, the results of grafting at distal levels are less definite than those at proximal levels because the incision alone is often effective as an organizer. This being the case, the part played by the graft in determining the new axis is not always certain. In view of the results obtained at proximal levels, it seems justifiable to assume that when the graft constitutes the distal end of the new axis it represents the primary factor in determining and organizing that axis. Unfortunately, in some cases stages early enough to permit determination of this point were not examined; but in most of those which were examined, the graft was found at the distal end of the new axis or of one of the axes which developed.

When the graft is within 10 mm. of the distal cut end of a stem 50 mm. or more in length, it is almost always included in the region which gives rise to the host hydranth; and even at 15 mm. from the cut end, it may be included in this region. In such cases it merely determines a duplication of more or less of the distal portion of the host hydranth (Figs. 16–18). Often, also, the distal end of the graft axis may itself be duplicated, as in Figures 17 and 18, in each of which the graft axis shows two hypostomes.

Figures 19–24 show further variations in the observed results. In Figure 19 the graft lies between two partial manubria, both of which develop from the host tissue. In this case the graft apparently did not act as organizer. It may have served merely as a means of



FIGS. 16–25.—Development of proximal pieces grafted at distal levels. Figure 16, a graft axis consisting of only distal part of manubrium. Figures 17 and 18, graft axes with two hypostomes. Figure 19, two new axes from host tissue with graft between them. In this case the graft evidently did not act as an organizer but apparently served as a mechanical means of keeping the incision open and so increasing cellular activity in connection with it. Figure 20, two new axes, one from graft, one from host. Figures 21 and 22, graft axes from proximal tentacle region of host, Figure 22 with two hypostomes from graft. Figure 23, one new graft axis, a second from host tissue in proximal tentacle region. Figure 24, a graft axis proximal to terminal hydranth. Figure 25, a graft in hydranth region without development of a new axis.

retarding closure of the incision, thereby increasing the cellular activation concerned in closure, for apparently a new axis developed from the incision on each side of the graft. In Figure 20 two axes, one distal to the other, have developed in the proximal tentacle region of the host hydranth. The graft tissue forms the apical end and the proximal side of the more proximal axis, but takes no direct part in the distal axis. In Figure 21 the graft axis arises at a slightly more proximal level of the host and develops a complete hydranth, the graft constituting the hypostome and the distal part of the manubrium. Figure 22 shows a case of development of two hypostomes, both of which arise from the graft, and a single manubrium and hydranth body. In Figure 23 two manubria with a single proximal hydranth region develop side by side, and the graft appears at the apical end of one of them. The new axis of Figure 24 is entirely proximal to the host hydranth and develops a stem of its own. In this case the hypostome and most of the manubrium develop from the graft. Figure 25 shows a characteristic case of failure to develop a new axis from a graft in the hydranth region. The graft is still visible as a protuberance on the side of the hydranth but is gradually undergoing resorption.

The lateral axes which develop from the incision in the controls for distal levels (33 per cent) range from hypostome and more or less of the manubrium to complete hydranth and stem, according to the level at which they appear; but in no case observed thus far has an incision without graft given rise to more than one new axis. In the axes developing from incisions with lacerated margins described in an earlier paper (Child, 1927*b*), duplication occasionally occurred; but with the incisions used as controls for the grafts, no duplication has been observed. Apparently the presence of the graft favors duplication, particularly in this distal region, probably because the graft, together with the incision, provides possibilities of more than one center of activity. For example, the graft may perhaps determine a new axis on both its distal and proximal surfaces; or it may serve, as it apparently does in the case of Figure 19, to separate two active regions of host tissue.

It is important to note that the portion of the new axis which develops at these distal levels, either from the incision alone or from

the graft, depends on the level of the host axis at which it arises. In the hydranth region of the host the new axis does not develop more than those parts of a hydranth corresponding to the parts of the host hydranth distal to the level of the new axis. If it develops distal to the proximal tentacles, it consists only of hypostome and manubrium or partial manubrium (Figs. 16–19). At the level of, or just proximal to, the proximal tentacles it usually develops a hydranth body including proximal tentacles but nothing further (Figs. 21, 22); and at still more proximal levels it develops a complete hydranth and more or less stem (Fig. 24) which gradually elongates and may become equal in length to that part of the original axis distal to the level at which the two axes join. In a few cases among the new axes developed in the proximal region of the original axis near the distal perisarcal boundary, the two stems gradually increase in length at the expense of the short common proximal region and finally separate with the development of basal ends on each. This appears to be a case of growth of the two distal regions at the expense of the common proximal region rather than a reorganization of the latter. Among the new axes developed from lacerated incisions (Child, 1927*b*), a few cases have been observed in which the new axis determined a new basal end from the side of the stem opposite that from which the distal end of this axis developed. In all such cases, however, the original basal end had been removed and the terminal hydranth had been repeatedly cut off as each new one developed. Under these conditions the old stem had undergone considerable shortening, showed little contractile activity, and was apparently at a distinctly lower physiological level than an attached erect stem.

In some cases the lateral axis developing from graft or incision apparently represents less than that portion of the original axis distal to its level of origin. In Figure 17, for example, the two new axes develop no further than distal tentacles, although they arise some distance proximal to the distal tentacles of the main axis. In Figure 20 the two manubria arising at the level of the proximal tentacles of the original axis develop only parts distal to the proximal tentacles. These axes did not develop more proximal levels at later stages.

IMPLANTATION OF PIECES IN THE ENTODERMAL PARENCHYMA

A few implantations of small stem pieces into the entodermal parenchyma were made in order to determine whether the pieces could live and undergo reorganization there and whether they could determine new axes, either as organizers directly concerned in their formation or possibly in some other way, e.g., through some general chemical action on the host tissues about them by products of necrosis or other substances. In these experiments the hydranth of the host stem was removed and the proximal end of the stem cut off at the perisarcal boundary. A needle was then thrust into the entodermal parenchyma longitudinally for a distance of $\frac{1}{2}$–1 cm. from the proximal cut end, and the graft was then pushed to the end of the canal thus formed and so was imbedded in the entodermal parenchyma at a level where the superficial regions of the stem were uninjured. In all of these cases, ten in number, the graft promptly underwent necrosis and was completely disintegrated within 2–3 days. No indication was found in any case of an effect of any sort on the superficial parts of the stem at the level concerned.

DISCUSSION

Grafts and incisions as organizers.—The experiments show that the graft of a small stem piece into distal or proximal stem levels may act as an organizer in the sense in which Spemann and his co-workers have used the term, that is, it may determine the development of a new axis almost entirely from host tissue. The graft differs from the amphibian organizer of normal development in that it need not represent a particular region or level of the stem and in that it constitutes the apical end of the new axis; but, as will appear below, these differences do not appear to be of fundamental significance. The graft in *Corymorpha* is able to appropriate or dominate considerable areas of the host tissue and to reorganize them into parts of a new axis. Apparently there is nothing specific in this reorganizing action of the graft. Pieces from both distal and proximal levels of the stem act as organizers, and presumably pieces from intervening levels possess the same capacity. Moreover, the actual implantation of grafts is not necessary for the organization of new axes. The experiments show that at distal levels of the stem a simple in-

cision with slight laceration of the entodermal parenchyma, which probably does not appreciably affect the result, may organize a new axis. At the proximal level such an incision has not been observed to act as an organizer, but, as already noted, incisions with sufficiently lacerated margins may give rise to organizing regions (Child, 1927*b*). It has also been shown that in the reconstitution of isolated pieces and of aggregates of dissociated cells development progresses from the apical end basipetally, except as regards the basal end, which may be determined by local external conditions to which a particular region of the piece or aggregate is subjected. In short pieces, as much of the axis develops from the apical end basipetally as the length of the piece, the scale of organization, and conditions at the opposite end or in other regions of the piece permit (Child, 1926*b*, 1927*a*, 1928*b*). Apparently in all these cases the portion of the piece which is determined as apical end dominates other regions to some extent and acts as organizer.

Apparently the original polarity of the implanted piece has little or no influence on the direction of the original axis. It has been shown in earlier papers (Child, 1926*b*, 1927*a*) that in short pieces of *Corymorpha* stem the original polarity is so slight that it is readily obliterated and a new axis determined by environmental factors which act differentially on different regions. The pieces used as grafts were even shorter than most of those of the earlier experiments, and they were implanted in any position. It is certain that a considerable number of them were in such position that the lateral surface was outward. So far as could be determined in such cases, this lateral surface became the new apical end. It is also certain that other pieces were implanted in other positions; but in the experiments thus far performed, no attempt has been made to distinguish distal and proximal cut surfaces of the graft. It is possible that in some cases of development of two axes from a graft these may arise from distal and proximal cut surface, respectively; but in the cases observed (Figs. 6, 22, and a few other similar cases not figured), the positions and relations of the two axes to each other make it appear more probable that they were determined simply by two regions of cellular activity arising in the course of the changes in the graft without reference to the original polarity. It has been shown re-

peatedly in earlier papers that multiplication of axes may occur in isolated pieces without reference to the original polarity (Child, 1926*b*, 1927*a*). In all cases observed the polarity of the new axis determined by the graft or by an incision is vertical or nearly vertical to the surface of the stem from which it arises. Apparently the graft, like the incision, organizes because it represents a region of localized cellular activity rather than because of its original polarity.

In the light of these various facts it seems to be evident that the organizer in *Corymorpha* is not a particular tissue entity but a physiological condition. All the evidence indicates that any region or part which can attain and maintain a sufficiently high level of intrinsic physiological activity may act as organizer. The simple incision at proximal levels does not organize, but the incision with lacerated margins does (Child, 1927*b*). At distal levels the simple incision without laceration of margins often acts as organizer. Apparently this greater effectiveness of the incision as organizer at distal levels results from the fact that this level represents a higher level of the gradient, and so of physiological activity, than the more proximal levels (Child, 1926*a*; Child and Hyman, 1926). At this level the reaction of the cells to the incision is adequate in many cases to localize a region sufficiently active to initiate the organization of a new axis. At the proximal levels additional laceration is necessary for this result. Attention is particularly called to the fact that the new axis determined by such an incision does not arise from the proximal margin of the open incision in the way that a partial disc and tentacles develop from a lateral incision in a sea anemone (Child, 1909). The incision closes within at most a few hours; and the new axis develops, not from a cut surface, but from the region of cellular activity localized by the incision. This region is not sharply bounded but apparently decreases radially from a central area. The radial gradient in activity becomes evident in the more rapid growth of the central area, and this gives rise to a localized lateral outgrowth with the most active region at its tip. In consequence of the outgrowth the radial gradient resulting from the localization of the active region becomes an axial gradient. There is no evidence and no good reason for believing that the physiological axis determined

by an incision in the stem of *Corymorpha* is primarily anything more than this.

The graft is more effective as an organizer than the incision alone, but there is nothing in the facts to indicate that this difference is not merely quantitative. Undoubtedly the reorganization of the graft involves greater physiological activity than the mere closure and healing of the incision. As already noted, the distal piece grafted at a proximal level is more effective than the proximal piece at the same level, apparently because the distal piece represents a more active level of the stem and reorganizes more rapidly. In short, the various lines of evidence agree in indicating that the ability of a region to organize a new axis depends primarily on quantitative factors involving the metabolism of the region rather than on specific constitution or activities. If this is true, it constitutes a new line of evidence in support of the view that the physiological axis is primarily a quantitative physiological gradient.

In *Hydra* a graft consisting of a small piece from the region at the base of the tentacles is capable of organizing a new axis (Browne, 1909), but grafts of similar size from more proximal levels do not act as organizers, though in large grafts from these levels the axis already present may persist. According to these experiments, *Hydra* differs from *Corymorpha* in that there only the higher levels of the gradient are capable of organizing new axes, while in *Corymorpha* proximal, as well as distal, levels of the stem possess this capacity in some degree, even when grafted into distal levels. It is suggested that this greater organizing capacity of the more proximal stem levels in *Corymorpha*, as compared with *Hydra*, is associated with the extremely rapid reconstitution of pieces from all stem levels. Under favorable conditions and at a temperature of about 20° C. the new individual, hydranth, or in small pieces the partial hydranth is advanced in development at 48 hours after isolation or grafting in *Corymorpha*. The hydranth is well formed, tentacles are often 2–3 mm. in length, and in isolated pieces a basal end may already be differentiated. In *Hydra* reorganization proceeds more slowly. It seems probable that this more rapid reorganization in *Corymorpha* involves a greater increase in physiological activity than occurs in *Hydra* and that the effectiveness as organizers of pieces of the stem,

even from the more proximal levels, is associated with this greater activity.

The parts determined by the organizer in relation to its level on the original axis.—Attention was called above to the fact that in no case observed thus far among the grafts and their controls does a new lateral axis arising in the hydranth region of the original axis develop levels proximal to the level of its origin. This is to be expected if the two organizing regions, that of the original and that of the new axis, represent about the same level of physiological activity. Under these conditions the new lateral axis will organize the various parts distal to the level of origin but will have no organizing effect to develop more proximal levels because they are already present. If its activity were much greater than that of the dominant region of the original axis, the organization might proceed farther proximally; and if it were less, organization would not proceed as far. Among the grafts and their controls no certain case of organization of more than the regions distal to the level of origin has been observed thus far, but in an earlier paper certain cases were described in which a lacerated lateral incision without graft not only determined hydranth and distal stem levels but also the development of a basal end from the lateral surface of the stem opposite that on which the incision was made and the distal end developed (Child, 1927*b*, Figs. 6, 8). In these cases the basal perisarcal region of the original stem was entirely removed, the terminal hydranth was cut off, and new hydranths developing terminally were also removed every 2 to 3 days. Under these conditions the organizing action of the new axis was effective across the original axis.

When the new axis arises from a level proximal to the hydranth of the original axis, it develops more or less stem (Figs. 8, 10, 24), and this may gradually elongate until it equals in length that part of the original axis distal to the level of union. When the common proximal region is short in relation to the two distal regions, these have been seen in a few cases to undergo a gradual further elongation at the expense of the common proximal region, which becomes shorter and shorter and finally disappears, and the two axes separate, each developing a basal end. The slowness of this process as compared with the development of the new axis suggests that it is

due to further development and growth of the two incomplete axes at the expense of the common proximal region rather than to actual reorganization of the latter.

Similar phenomena have been observed by various investigators in *Hydra*, both in cases of lateral grafts and of the formation of two distal regions as the result of partial longitudinal splitting, and have sometimes been described as a migration of one axis down the other. In this change of position, however, the distal regions of both axes become longer and apparently develop the more proximal regions, while the common proximal region becomes shorter. Moreover, Browne (1909) has shown that when a graft axis and host axis are of different color each maintains its original color during the process of elongation and separation, except perhaps for a few cells in the region where separation occurs; that is, neither component takes over bodily cells of the other, but apparently each grows and develops at the expense of the common proximal region. Probably the process of gradual separation of the two components in *Corymorpha* is essentially similar. By way of interpretation it may be suggested that a complex consisting of two distal regions and one proximal is to some degree out of proportion physiologically, since the amount of the more active tissue of the distal levels is relatively much greater than in the normal individual. If the duplicated distal regions are short compared with the common proximal portion, the disproportion is slight and growth of the distal regions at the expense of the proximal may be slow or may not occur at all, according to the species. But if the distal regions represent a large part of the axis, they may be able to continue their development at the expense of the short proximal region and so finally to form proximal parts and separate. That size is a factor in maintenance or further growth of a portion of an axis appears clearly in the experiments on grafting in *Hydra*. The larger grafts from regions proximal to the tentacles are less frequently resorbed than the smaller.

As regards the naked stem region of *Corymorpha*, there seems to be no question that the primary difference between an organizer and other parts is essentially a quantitative one and that any region of the stem which becomes sufficiently active in relation to adjoining parts may serve to some extent as an organizer. Whether parts of

the hydranth which have undergone more or less specialization will function as organizers remains to be determined.

The organizer in amphibian development.—The researches of Spemann and his associates on urodele development have led to the conception of the *Organisator* or organizer as an important factor in amphibian development. The recent general discussions of the amphibian organizer by Spemann and others (Spemann, 1925, 1927; Mangold, 1926; De Beer, 1927) make it unnecessary to consider the earlier literature of the subject at this time, but it is a question of considerable interest whether the amphibian organizer is something fundamentally different from the organizers in *Corymorpha*, that is, a specific tissue entity, or whether it is primarily a region of high physiological activity, such as the organizers in *Corymorpha* appear to be. The region first identified as organizer in urodele development is the dorsal lip region of the blastopore. The earlier experiments indicated that the differentiation of the medullary plate is dependent on the presence of this region or part of it beneath the ectoderm of the medullary plate region. In normal development the organizer attains this position as the result of gastrulation, but further experiment has shown that pieces of the organizer region implanted in the blastocoel are effective in determining medullary plate development and even in determining a second embryonic primordium in another region when one was already present. Recent investigation along this line has added many facts which throw light on the problem of the nature of the organizer. Geinitz (1925), for example, has shown that the organizer is not only not species-specific in its action but not even order-specific. Mangold and Spemann (1927) have found that medullary plate material after its determination as such may itself serve as organizer, irrespective of the amount of archenteron material attached to it. Later Spemann and Geinitz (1927) found that ectoderm transplanted into the organizer region acquired the capacity to act as organizer and that even this secondarily determined organizer is not species-specific. Bautzmann (1926) has shown by implantation in the blastocoel of pieces from definite regions with respect to the blastopore that the whole quadrant above the blastopore and to some extent also lateral and ventral regions are capable of acting as organizer. More

recently he has carried his analysis further in investigating the organizing capacity of chorda and mesoderm material (Bautzmann, 1928). He has found that chorda material develops organizing capacity at the blastula stage and loses it at the neurula stage, about the time when the medullary plate material develops it. The mesoderm possesses little organizing capacity as compared with the chorda. Mangold (1928) has recently reported experiments with various embryonic tissues and has found that parts of the brain of older embryos possess some degree of organizing capacity but less than the medullary plate material, and that limb buds transplanted into the blastocoel probably possess some slight degree of organizing action. Pieces of gill and of intestine similarly implanted do not function as organizers.

From the facts at present available it appears then, first, that the ability to function as organizer is not associated with the specific protoplasmic constitution of a particular species, that is, it is not species-specific and not even order-specific; second, that it is not present in a particular part at all stages but only during a certain period of development, which is different for different parts; third, that it may be present to a greater or less degree in different regions or tissues at different stages. In the light of these facts there seems to be no good reason for believing that the ability to function as organizer is associated with specific factors in the part which so functions. The experimental data seem to provide no basis for any other than a quantitative conception of the amphibian organizer. Goerttler (1927) has already suggested that the organizer differs from other parts of the embryo quantitatively rather than qualitatively, and that its influence is dynamic rather than material. The more recent experimental data provide further support for this view.

In short, at present the amphibian organizer appears to be primarily a region of high physiological activity which influences and determines other less active regions through this activity. Moreover, if the primary difference between it and other parts is quantitative, its influence is probably exerted by means of transmitted energy changes rather than by the mass transport of substance. If it is simply a more active region, it cannot produce substances which will affect other parts in a specific way; and the fact that it is effec-

tive even in another order of Amphibia certainly favors the view that its action is not specific. Apparently the amphibian organizer is a physiologically dominant region essentially similar to other dominant regions in that its dominance depends primarily on a quantitative rather than a specific difference from the parts which it dominates (Child, 1924, chap, x).

The question of the relation between the normal organizer region and the physiological gradients in amphibian development requires brief consideration. As regards *Triton*, Goerttler (1925) has found that the extreme anterior boundary of presumptive medullary plate material lies at the beginning of gastrulation, somewhat in front of the animal pole, and that the material from which the medullary plate normally develops has already undergone some degree of determination, so that a medullary primordium may develop without formation of an archenteron, that is, supposedly without the influence of the organizer, or at least without its presence beneath the medullary plate region. On the other hand, it has been observed that embryos induced by implantation of pieces of the organizer region are often defective at the anterior end. Lehmann (1926, 1928) has also concluded that the extreme anterior end may undergo at least a labile determination independently of the presence of the organizer beneath it, and that the degree of this determination decreases and the determining influence of the organizer increases in the posterior direction.

Some years ago Bellamy (1919) showed the presence of a primary region of high susceptibility about the animal pole of the frog's egg, even before cleavage, and a zone of higher susceptibility than that of other regions extending basipetally along the meridian of the gray crescent and decreasing in the basipetal direction. He also found that as gastrulation approaches, the susceptibility of the prospective dorsal lip region increases so that at the beginning of gastrulation this appears as a second region of high susceptibility and becomes the region of highest susceptibility in the embryo. The normal organizer of *Triton* corresponds closely as regards localization to this secondary region of high susceptibility in the frog's egg; and Brachet (1923, 1927) has found that the middle region of the gray crescent of the frog's egg acts to some extent as an organizer

but that the frog's egg is much less plastic than that of *Triton*. Bellamy's observations on susceptibility and on developmental modification indicate that the primary gradient from the apical pole basipetally is slight and the dominance of the apical region weak and of short range. If the differences in susceptibility observed by him result from quantitative differences in physiological condition, as many lines of evidence indicate (Child, 1928*c*, pp. 450–63), the secondary region of high susceptibility becomes the most active region of the embryo at or before the time of gastrulation. From the experiments on invertebrates we should expect such a region to be dominant to a greater or less extent in further development. Bellamy's data indicate, however, that the primary dominant region in the frog is about the apical pole and that the dorsal lip region becomes a highly active region only secondarily. Bellamy suggested that the appearance of the second region of high susceptibility might be the result of physiological isolation from the dominance of the primary apical region. Under inhibiting conditions he found in some embryos a secondary invagination anterior to the normal one but in the same meridian and similar in appearance (Bellamy, 1919, pp. 341–43, 351, Figs. 14*C*, 18), and suggested that such a secondary invagination might perhaps result from the physiological isolation of a region nearer the apical pole than the normal dorsal lip region in consequence of the decrease in range of dominance of the primary apical region under the conditions of experimental inhibition.

We have no data concerning differential susceptibility in the early developmental stages of *Triton;* but the similarity between it and the frog, as regards general course of development, makes it probable that there is no very great difference in the regional differences in susceptibility in the early stages. The observations of Goerttler (1925) and Lehmann (1926, 1928) concerning the position of the extreme anterior end of the embryo and its independence of the organizer agree well with Bellamy's data on susceptibility in the frog. Both lines of investigation indicate that the primary region of dominance is the region of the apical pole and that conditions there may organize the extreme anterior end of the medullary plate. The experimental data on the organizing action of the dorsal lip

region at the beginning of gastrulation indicate that in *Triton*, as in the frog, this region becomes active early in development and then constitutes the chief region of dominance in further development. A secondary dominant region appears in other segmented animals, so far as examined, e.g., in annelids (Hyman, 1916), in fishes (Hyman, 1921, 1926), and in the chick (Hyman, 1927; Hinrichs, 1927), and in the annelids which continue to grow throughout life it persists.

It appears then that the various lines of evidence agree in indicating, though they do not demonstrate, that the amphibian organizer does not differ fundamentally as regards the physiological basis of its action from the graft or the incision, or the normally dominant apical region in *Corymorpha*, or from dominant regions in their simplest forms in other organisms, both plant and animal. The data on the amphibian organizer are in full agreement with the gradient theory and constitute additional evidence in support of it. That fields of investigation so widely separated as amphibian ontogeny and the development and reconstitution of the lower invertebrates suggest similar conclusions is good ground for believing that real progress is being made in these fields.

If it is true that organizers in general are primarily regions of more intense physiological activity, it follows that the mechanism of orderly development and integration is primarily a quantitative mechanism and that specific relations of parts dependent on the production of specific substances are a secondary result of specialization. Moreover, according to this view we may expect to find organizers or dominant regions, not merely for the whole embryo, but in later stages for particular minor axes and parts. The conception of the *Organisationsfeld* or *Determinationsfeld* and *Wirkungskreis* (Weiss, 1926) does not differ fundamentally from the conception of dominance and subordination which constitutes a part of the gradient theory. Weiss, however, does not consider the problem of the physiological character of the dominant region and the nature of its action.

From what has been said, it appears that organizer and dominant region are essentially the same. It is becoming more and more evident that only with some such conceptions as these can we ac-

count for the orderly character of development and reconstitution and for the integration of the individual organism into a physiological whole.

SUMMARY

1. Grafts of very small pieces of the naked stem region of *Corymorpha palma* into lateral incisions in the naked stem may act as organizers and determine new axes of which the grafts themselves form only the most distal parts, other levels being developed under the influence of the graft from the host tissue.

2. Grafts from a proximal level just distal to the perisarc implanted at the same level are about 40 per cent less effective as organizers than grafts from distal levels implanted at the proximal level.

3. The new axes determined by the proximal grafts at proximal levels also develop more slowly, and the hydranths are organized on a smaller scale, than those determined by distal grafts at proximal levels.

4. At proximal levels the simple incision without graft has not determined a new axis in any observed case, but an incision with lacerated margins may organize a new axis at these levels.

5. At distal levels of the stem the incision alone may determine a new axis, but the presence of a graft from a proximal stem level increases the frequency of new axes nearly 60 per cent, though in some cases the graft apparently does not act as organizer, but merely as a means of retarding the closure of the incision and so increasing the activation of cells about it.

6. The portion of the axis organized by the graft or the incision depends on its level on the original axis. If the new axis develops in the hydranth region of the original axis, it gives rise only to those levels distal to the level of the original axis at which it arises and in some cases to only the more distal of these. If it arises near the proximal end of the stem, both it and that portion of the original axis distal to the level of union of the two sometimes increase slowly in length at the expense of the short common proximal region until both develop basal ends and separate. It is suggested that such gradual elongation and completion of the axis is not a reorganization of the cells of the common proximal region but a growth and de-

velopment of the distal regions at their expense, and that it results from a physiological disproportion.

7. The facts are regarded as indicating, first, that the organizer in *Corymorpha* does not represent a specific tissue entity but a physiological condition, and second, that the organizer differs primarily only quantitatively from other parts, that is, in the degree of physiological activity. Grafts from more active distal levels and incisions at such levels are more effective as organizers than grafts from, or incisions at, proximal levels; but apparently any level of the stem, when made sufficiently active, may become an organizer. These facts are in full agreement with the gradient theory and constitute new evidence in support of it.

8. The results of recent investigation concerning organizers in amphibian development are briefly discussed and it is pointed out that, according to the evidence at hand, amphibian organizers, like those in *Corymorpha*, appear to be primarily not specific entities but highly active regions. Apparently any region which is, or becomes, the seat of sufficient activity may function as an organizer.

LITERATURE CITED

BAUTZMANN, H. 1926. Experimentelle Untersuchungen zur Abgrenzung des Organisationszentrums bei *Triton taeniatus*. Arch. f. Entwicklungsmech., CVIII, 283.

———. 1928. Experimentelle Untersuchungen über die Induktionsfähigkeit von Chorda und Mesoderm. *Ibid.*, CXIV, 177.

BELLAMY, A. W. 1919. Differential susceptibility as a basis for modification and control of development in the frog. Biol. Bull., XXXVII, 312.

BRACHET, A. 1923. Recherches sur les localisations germinales et leur propriétés ontogénétiques dans l'œuf de *Rana fusca*. Arch. Biol., XXXIII, 343.

———. 1927. Étude comparative des localisations germinales dans l'œuf des amphibiens urodèles et anoures. Arch. f. Entwicklungsmech., CXI, 250.

BROWNE, ETHEL M. 1909. The production of new hydranths in *Hydra* by the insertion of small grafts. Jour. Exp. Zoöl., VII, 1.

CHILD, C. M. 1909. Factors of form regulation in *Harenactis attenuata*. II. Aboral restitution, heteromorphosis and polarity. *Ibid.*, p. 65.

———. 1924. Physiological foundations of behavior (New York), chap. x.

———. 1926*a*. Studies on the axial gradients in *Corymorpha palma*. II. Differential susceptibility, penetration and oxidation-reduction in development and reconstitution. Biol. Gen., II, 609.

CHILD, C. M., 1926*b*. Studies on the axial gradients in *Corymorpha palma*. III. Control and modification of polarity and symmetry in reconstitution by differential exposure. *Ibid.*, p. 771.

———. 1927*a*. Modification of polarity and symmetry in *Corymorpha palma* by means of inhibiting conditions and differential exposure. I. Forms resulting from modification. Jour. Exp. Zoöl., XLVII, 343.

———. 1927*b*. Experimental localization of new axes in *Corymorpha* without obliteration of the original polarity. Biol. Bull., LIII, 469.

———. 1928*a*. Physiological polarity and dominance in the holdfast system of *Corymorpha*. *Ibid.*, LIV, 15.

———. 1928*b*. Axial development in aggregates of dissociated cells from *Corymorpha palma*. Physiol. Zoöl., I, 419.

———. 1928*c*. Sammelreferat. The physiological gradients. Protoplasma, V, 447.

CHILD, C. M., and HYMAN, L. H. 1926. Studies on the axial gradients in *Corymorpha palma*. I. Respiratory, electric and reconstitutional gradients. Biol. Gen., II, 355.

DE BEER, G. R. 1927. The Mechanics of vertebrate development. Biol. Rev., II, 137.

GEINITZ, B. 1925. Embryonale Transplantation zwischen Urodelen und Anuren. Arch. f. Entwicklungsmech., CVI, 356.

GOERTTLER, K. 1925. Die Formbildung der Medullaranlage bei Urodelen. *Ibid.*, p. 503.

———. 1927. Die Bedeutung gestaltender Bewegungsvorgänge beim Differenzierungsgeschehen. *Ibid.*, CXII, 516.

HINRICHS, MARIE A. 1927. Modification of development on the basis of differential susceptibility to ultra-violet radiation. Jour. Exp. Zoöl., XLVII, 309.

HYMAN, L. H. 1916. An analysis of the process of regeneration in certain microdrilous oligochaetes. *Ibid.*, XX, 99.

———. 1921. The metabolic gradients of vertebrate embryos. I. Teleost embryos. Biol. Bull., XL, 32.

———. 1926. The metabolic gradients of vertebrate embryos. II. The brook lamprey. Jour. Morph. and Physiol., XLII, 111.

———. 1927. The metabolic gradients of vertebrate embryos. III. The chick. Biol. Bull., LII, 1.

LEHMANN, F. E. 1926. Entwicklungsstörungen in der Medullaranlage von *Triton* erzeugt durch Unterlagerungsdefekte. Arch. f. Entwicklungsmech., CVIII, 243.

———. 1928. Die Bedeutung der Unterlagerung für die Entwicklung der Medullarplatte von *Triton*. *Ibid.*, p. 123.

MANGOLD, O. 1926. Über formative Reize in der Entwicklung der Amphibien. Die Naturwissenschaften., Jhg. XIV, 1169.

———. 1928. Neue Experimente zur Analyse der frühen Embryonalentwicklung des Amphibienkeims. *Ibid.*, Jhg. XVI, 387.

MANGOLD, O., and SPEMANN, H. 1927. Über Induktion von Medullarplatte durch Medullarplatte im jüngeren Keim, ein Beispiel homöogenetischer oder assimilatorischer Induktion. Arch. f. Entwicklungsmech., CXI, 341.

SPEMANN, H. 1925. Some factors of animal development. Brit. Jour. Exp. Biol., II, 493.

———. 1927. Organizers in animal development. Proc. Roy. Soc., Ser. B., CII, 177.

SPEMANN, H., and GEINITZ, B. 1927. Über Weckung organisatorischer Fähigkeiten durch Verpflanzung in organisatorische Umgebung. Arch. f. Entwicklungsmech., CIX, 129.

WEISS, P. 1926. Morphodynamik. Abhandlungen z. theoret. Biol., H. 23, pp. 1–43.

EXCRETION OF NITROGENOUS SUBSTANCES IN PROTOZOA[1]

(Four figures)

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I. NATURE OF NITROGENOUS END-PRODUCTS OF METABOLISM IN PROTOZOA

THE nitrogenous end-products of metabolism in organisms vary in their nature according to the type of organism and its physiological condition. For example, in man the bulk of the nitrogen is eliminated as urea, and considerably less as uric acid, amino acids, and other nitrogenous compounds; while in birds and reptiles the bulk of the nitrogen is eliminated as uric acid. The question then arises: In what form is nitrogen excreted by Protozoa —as ammonia, urea, uric acid, or other substances, or a combination of these?

Griffiths (1888) concludes that the contractile vacuoles of certain Protozoa perform the function of a kidney and that their secretions are "capable of yielding microscopic crystals of uric acid." In his experiments he used *Amoeba sphaerococcus* (Haeckel), *Vorticella* sp., and *Paramecium bursaria*. A number of the organisms were subjected to the murexide test. The development of reddish-purple color indicated the presence of uric acid. In describing these experiments, Griffiths says (p. 132): "After the addition of alcohol minute flakes could be distinctly seen floating in the fluid of the vacuoles. Bearing in mind the murexide reaction, there is every reason to believe that these flakes are nothing more or less than minute crystals of uric acid." These experiments were repeated many times, generally with positive results, indicating the presence

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of uric acid. At times, however, the vacuole was found not to contain the slightest trace of uric acid.

Howland (1924) repeated these experiments, using several specimens of *Centropyxis* and *Amoeba verrucosa*. No crystals of uric acid were found in the distended vacuoles after the addition of alcohol; nor was the characteristic murexide reaction for uric acid obtained after the addition of ammonia, either in the vacuoles or in the culture medium immediately surrounding the organism. These experiments were conducted over a period of 5 weeks, always with negative results. However, by making use of the Benedict blood-filtrate test, Howland demonstrated the presence of uric acid in cultures of *Amoeba* and *Paramecium*. In this test the presence of uric acid is evidenced by the development of blue color, the intensity of the color increasing with a corresponding increase in concentration of uric acid. In Howland's experiments the depth of the color varied with the age of the cultures, the older ones giving a deeper color. From this she concluded that these organisms excrete uric acid.

Adolph (1922) concludes that "either waste-products are not deleterious through their retarding of metabolism, or urea is not an excretory product in *Phagocata* and *Paramecium*."

These observations appear to offer the only direct evidence concerning the nature of nitrogenous end-products in Protozoa. Since the evidence is so limited, and since that which exists is not wholly in agreement, the question cannot be considered definitely answered.

MATERIAL AND METHODS

In an effort to answer this question the following experiments were designed. A large number of organisms—*Paramecia*, *Spirostoma*, or *Didinia*—were thoroughly washed, placed in modified Ringer's solution,[1] allowed to remain for a time, and then removed by filtration. The filtrate was tested for various nitrogenous substances according to methods described below.

The *Paramecia* were washed as follows: Culture fluid containing the organisms was poured into a long-necked bottle of approximately 1 liter capacity until it was filled to within about 5 cm. of the top. Then tap water was added carefully to avoid mixing, until the bottle was entirely full. Within 5 or 10 minutes under these conditions

[1] $CaCl_2$ 2.22 grams, KCl 0.178 grams, NaCl 2.24 grams, water to make 30 liters.

the *Paramecia* usually aggregate in great numbers near the surface of the water. When they had thus collected at the top of the bottle, the surface water containing the *Paramecia* was removed with a pipette, and more tap-water added. This process was repeated, usually three or four times, until most of the organisms had been removed from the culture fluid. The bottle was now emptied, and the water containing the *Paramecia* put into it; then it was filled with tap-water and left until the animals came to the surface, after which they were removed as before. The *Paramecia* were thus washed in tap-water three or four times, after which they were usually found to be free from all heavy débris and large bacterial masses. Smaller organisms which were removed with the *Paramecia* were separated from the *Paramecia* by further washing on filter paper. The paper was about 20 cm. in diameter, and contained pores small enough to retain the *Paramecia* but large enough to allow the smaller organisms to pass through. About 2 liters of water, more if necessary, were used for this part of the washing process. The last part of this wash-water was the modified Ringer's solution previously mentioned. This solution was used so that a uniform medium of known composition might be used for all the various experiments. The animals seem to live very satisfactorily in it. The *Paramecia* were then transferred to a clean beaker, and the number per cubic centimeter ascertained by counting those in several measured volumes and averaging the results. In the experiments on *Paramecium* the numbers range from 1,000 to 3,500 per cubic centimeter. That the animals were sufficiently washed is evidenced by the fact that tests for various nitrogenous substances made immediately after the completion of this procedure were invariably negative.

Neither *Spirostomum* nor *Didinium* aggregates in the upper part of the solution as readily as *Paramecium*, so the washing process had to be limited to such as could be accomplished on filter paper. Since negative results were obtained in tests for the various nitrogenous substances, which were made immediately after washing was completed, it is concluded that washing in this manner was sufficient. The numbers per cubic centimeter were ascertained as for *Paramecium*. There were from 300 to 1,000 *Spirostoma* and from 160 to 500 *Didinia*.

After the organisms had been washed as described above, they

were allowed to remain in the modified Ringer's solution for various periods of time. At intervals samples of the solution were removed, filtered, and the filtrate subjected to chemical tests for ammonia, urea, uric acid, creatin, and creatinin as follows:

Ammonia by direct Nesslerization.—In the presence of ammonia Nessler's reagent gives a distinctive reddish-brown color, which varies in intensity according to the concentration of ammonia. By comparing the intensity of color produced in a solution of ammonia of unknown concentration to that produced in a solution of ammonia of known concentration this test may be used as a quantitative indicator.

Urea by hydrolysis to ammonium carbonate by means of urease,[1] *and subsequent determination of ammonia thus produced by Nesslerization.*—In so far as it is known, urease is specific in its action on urea.

Uric acid by Benedict blood-filtrate test.—Five cc. of the unknown solution are treated with 1 cc. of a 5 per cent solution of sodium cyanid in order to intensify the color produced by a modified phosphotungstic acid reagent. The chief modification by Benedict of the original Folin method is the introduction of arsenic pentoxid to form an arsenophosphotungstic acid reagent, which increases its specificity for uric acid.

Creatin by converting it to creatinin by hydrolysis, and testing for creatinin.—To one volume of the unknown add an equal volume of a 10 per cent solution of hydrochloric acid. The mixture is then placed in an autoclave for 10 minutes at a temperature of 115° C.

Creatinin by Jaffe's test.—To 5 cc. of the unknown solution add 1 cc. of a saturated solution of picric acid and 5 drops of a 10 per cent solution of sodium hydroxide. If the unknown contains creatinin, the solution gradually becomes reddish owing to the formation of creatinin picrate.

Creatinin by Weyl's test.—To 5 cc. of the unknown solution add 5 drops of a 5 per cent solution of sodium nitroferrocyanid (nitroprussid) and a few drops of a 5 per cent solution of sodium hydroxide.

[1] Urease-Dunning, prepared by Hynson, Westcott, and Dunning, Baltimore, Maryland.

The appearance of a permanganate color indicates the presence of creatinin.

RESULTS

The results of the analyses of the filtrates are presented in Table I. This table indicates that *Paramecium* excretes urea, but no ammonia, uric acid, creatin, or creatinin. Solutions in which *Paramecia* were allowed to remain for several hours gave a decidedly positive test for ammonia with Nessler's reagent after hydrolysis with urease. No evidence of ammonia was obtained before hydrolysis. Urea, then, must have been hydrolyzed with the subsequent production of the ammonia found after the addition of urease.

TABLE I

NITROGENOUS EXCRETORY PRODUCTS OF *Paramecium*, *Spirostomum*, AND *Didinium*

Organism	Ammonia	Urea	Uric Acid	Creatin	Creatinin
Paramecium	none	some	none	none	none
Spirostomum	none	some	none	none	none
Didinium	some	none	trace?	none	none

In tests in which the *Paramecia* remained in the solution comparatively long (12 hours, or more), a positive indication of ammonia was often found before the addition of urease. This ammonia undoubtedly arose from hydrolysis of urea by bacteria which had accumulated in the solution. That the bacteria which are found in *Paramecium* cultures hydrolyze urea was demonstrated in the following manner. A dilute solution of urea was inoculated with culture fluid. After the solution had been allowed to stand for several hours, it gave a positive indication of ammonia with Nessler's reagent. Furthermore, the length of time required for ammonia to make its appearance was not dependent upon the number of *Paramecia* present. If excretion by the *Paramecia* had been the source of this ammonia, a direct relation between these two factors should have been evident. Ammonia appeared only after the experiment had been in progress a considerable length of time. The methods for washing the *Paramecia* were such that a minimum of 12 hours was necessary for the bacteria which had not been removed to multiply to such an extent as to hydrolyze enough urea to give a positive reaction with Nessler's reagent.

Essentially the same results were obtained with *Spirostomum* as with *Paramecium*. Urea was the only nitrogenous substance found.

In tests with *Didinium*, however, a very definite indication of ammonia was obtained without the use of urease; and the depth of color developed after the addition of this enzyme did not appear greater than that produced in another portion of the same sample to which it had not been added. This was taken to indicate that no urea is excreted by this form. If the organisms were allowed to remain in the solution for as long as 24 hours, a trace of uric acid was sometimes found, but the quantity was so slight as to make impossible an estimate with any considerable degree of accuracy. No evidence whatever was obtained of the presence of creatin or creatinin.

As previously stated, the tests for uric acid in experiments with *Paramecium* and *Spirostomum* were negative at all times. In one experiment 2,800 *Paramecia* per cubic centimeter of solution were allowed to remain in the modified Ringer's solution for 72 hours; in another, 2,500 per cubic centimeter for 6 days. In neither of these experiments, and in none of those in which the length of time was shorter, was any indication of uric acid obtained. These results were unexpected in view of the fact that Griffiths claimed to have seen crystals of uric acid in the vacuoles of *Amoebae*, and Howland gave conclusive evidence concerning the presence of uric acid in cultures of Protozoa. Griffiths' experiments, however, were not confirmed by Howland although she followed his technique specifically. But the presence of uric acid in mass cultures has been repeatedly confirmed by the author. The question then arises as to the source of this substance.

The author demonstrated that uric acid is not eliminated by *Paramecia* or *Spirostoma* kept in modified Ringer's solution or tap-water, but he almost invariably found it in samples taken from ordinary cultures. Furthermore, the quantity, according to Howland, increases with the age of these cultures. If all these statements are valid, then the uric acid found in the culture fluid must have originated in substances from which the fluid was made. Various substances commonly used in the preparation of culture media—hay, wheat, barley, rye, oats, malted milk, beef extract, blood fibrin, and blood albumen—were therefore tested for uric acid. Without

a single exception, a cold aqueous solution or extract of each of these substances yielded with the Benedict blood-filtrate test unmistakable evidence of uric. The fact that Howland found that old cultures gave more strongly positive tests may be explained in the following manner. As the cultures become older, more and more uric acid dissolves from the material used in the preparation of the medium. The solubility of uric acid in cold water is comparatively slight, which would tend to produce a slow rate of diffusion from the substance which contains it, and a corresponding increase in concentration with the passage of time. Or, another conceivable source of uric acid which would give an increased concentration with the passage of time is the decomposition of proteins in the culture medium.

II. RATE OF EXCRETION OF NITROGENOUS END-PRODUCTS OF METABOLISM IN PROTOZOA

In 24 hours the average adult human male excretes about 15 grams of nitrogen (Morse, 1927, p. 696). This amount varies greatly according to the physiological condition—hunger, nature of diet, etc. For the fourteenth day of starvation the amount eliminated is only about half what it is in well-fed individuals; i.e., 7.7 grams (Morse, 1927, p. 568). So far as the author has been able to ascertain, there is no evidence concerning the rate of excretion in Protozoa, or concerning the effects of starvation and satiety on the rate of excretion. With these facts in mind experiments were designed which have a direct bearing on these questions.

MATERIAL AND METHODS

In these experiments the three forms previously mentioned—*Paramecium*, *Spirostomum*, and *Didinium*—were used. The rate of excretion of nitrogenous substances was ascertained in the following manner: The organisms were washed according to methods previously described, and put into modified Ringer's solution. At appropriate time intervals samples of the fluid were removed and the quantities of nitrogenous substances contained therein measured by methods described in a preceding section. In these experiments various numbers of organisms were used, but the results were calculated on the basis of the following numbers per cubic centimeter for

the different species: *Paramecium*, 2,500; *Spirostomum*, 500; and *Didinium*, 200.

RESULTS

The results obtained in these experiments are presented in Tables II, III, and IV and in Figures 1, 2, 3, and 4.

Paramecium.—By referring to Table II and Figure 1, it will be seen that the amounts of ammonia produced by hydrolysis of the

TABLE II

RATE OF EXCRETION OF UREA IN *Paramecium**

Exp. No.	No. of Animals per Cc.	Time of Washing (Hr.)	Ammonia: Parts per 1,000,000 by Weight for 2,500 Animals per Cc. Excreted in—								
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.	12 Hr.
3	1,000	1	0.87	1.20	1.20		1.20				
4	2,500	½	0.82	1.17	1.17	1.17				1.17	
7	2,000	½	0.90	0.98	1.10	1.15		1.25			
8	2,200	1	0.77	0.90			0.90	17 hr. 0.90			
Average			0.84	1.06	1.16	1.16	1.05				
1	2,500	1				1.80				1.90	2.00
2	1,000	½		1.65		1.95				1.95	2.20
5	2,500	1	0.65	1.15	1.57	1.75				1.75	
6	1,500	½		1.02			1.82			2.00 (17 hr. 2.00)	
Average			0.65	1.27	1.57	1.83	1.82			1.90	

* The amount of urea excreted by 2,500 animals per cubic centimeter of solution in 1–12 hours is given in parts of ammonia by weight per 1,000,000 of solution after hydrolysis. To convert parts of ammonia to parts of urea, multiply figures given by 1.76. Note that the rate of excretion is nearly zero after 2 hours.

urea present at successive periods, when plotted as ordinates against time as abscissae, form curves of a fairly uniform type; and that these curves rise at a nearly constant rate to a point indicating a concentration of urea sufficient to produce 0.90–1.25 parts by weight of ammonia per 1,000,000 of solution after hydrolysis, and then become practically horizontal. This indicates that excretion continued at a uniform rate during the first 1 or 1½ hours, and then ceased entirely or fell to a very low rate. Essentially the same thing

is shown in Table II and Figure 2. The curves in this figure indicate, however, that excretion continued until sufficient urea had accumulated to produce, after hydrolysis with urease, a concentration of

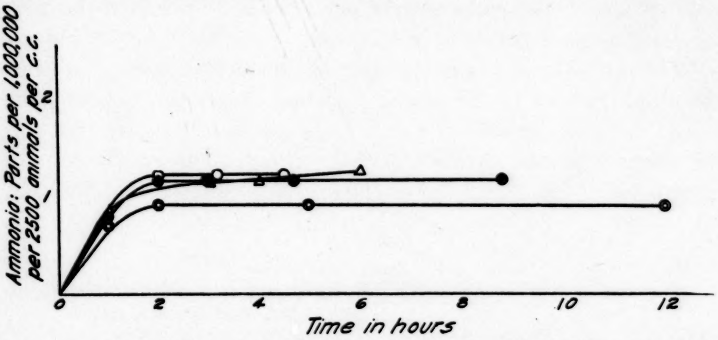

FIG. 1.—*Paramecium:* concentration of urea in surrounding medium at successive periods of time as indicated by the amount of ammonia produced by hydrolysis. ○, Exp. 3; ●, Exp. 4; △, Exp. 7; ⊙, Exp. 8.

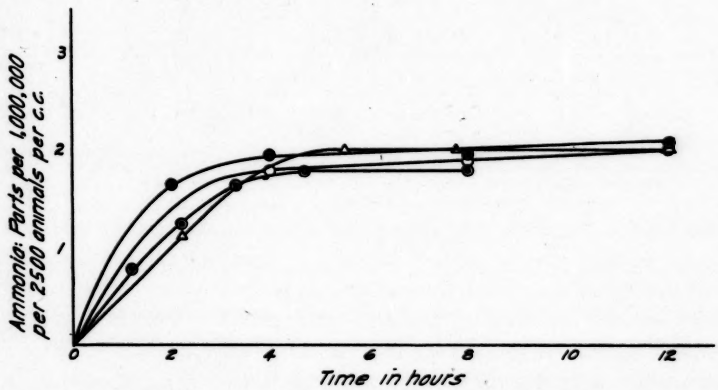

FIG. 2.—*Paramecium:* concentration of urea in surrounding medium at successive periods of time as indicated by the amount of ammonia produced by hydrolysis. Amount of ammonia produced by hydrolysis at successive periods of time. ○, Exp. 1; △, Exp. 2; ⊙, Exp. 5; ●, Exp. 6.

ammonia of about 1.75–3.10 parts by weight per 1,000,000 of solution, requiring from about $2\frac{1}{2}$–4 hours before the rate of excretion fell to a minimum.

From the slope of the curve it is possible to compute the rate of excretion at any period. Thus, during the first hour represented in Figure 1, and the first 2 hours in Figure 2, the rate of excretion was approximately 1.40 parts of urea per 1,000,000 by weight per hour for 2,500 animals per cubic centimeter.

The question now arises as to what factors are correlated with the rapid decrease in the rate of excretion observed. As previously stated, in man the rate of excretion is specifically correlated with the amount of food available. Observations made on the relative abundance of food vacuoles in *Paramecium* indicate that this also

TABLE III

RATE OF EXCRETION IN *Spirostomum**

Exp. No.	No. of Animals per Cc.	Time of Washing (Min.)	Ammonia: Parts per 1,000,000 by Weight for 500 Animals after—							
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.
1.....	275	20		1.70		2.55				2.55
2.....	425	20		2.00		3.00				3.00
3.....	350	15	0.80	1.05	1.87		2.85			2.85
4.....	400	15	1.00	1.17		1.90				1.90
Average			0.90	1.48	1.87	2.48	2.85			2.58

* The amount of urea excreted by 500 animals per cubic centimeter of solution in 1–8 hours is given in parts of ammonia by weight per 1,000,000 of solution after hydrolysis. To convert parts of ammonia to parts of urea, multiply figures given by 1.76.

obtains for Protozoa. In these observations it was found that the food vacuoles were abundant at the beginning of the experiments but that after starvation for several hours they were entirely or almost entirely lacking. Coincident with the disappearance of the food vacuoles was the change in direction of the curve, indicating a decrease and finally the cessation of excretion.

Spirostomum.—Essentially the same type of phenomena were observed in the experiments on *Spirostomum* as in *Paramecium*. The initial rate of excretion was found to be approximately 1.25 parts of urea by weight per 1,000,000 of solution per hour for 500 animals per cubic centimeter. This rate of excretion continued for 3–4 hours, after which it decreased to a very low figure or to zero (Table III and Fig. 3).

Didinium.—Experiments on *Didinium* yielded more direct evidence concerning the effects of starvation and satiety on the rate of excretion than was obtained in the observations on *Paramecium.* As previously stated, *Didinium* excretes ammonia, whereas *Paramecium* and *Spirostomum* excrete urea. Four tests were made to ascertain the rate of excretion of ammonia in *Didinium* according

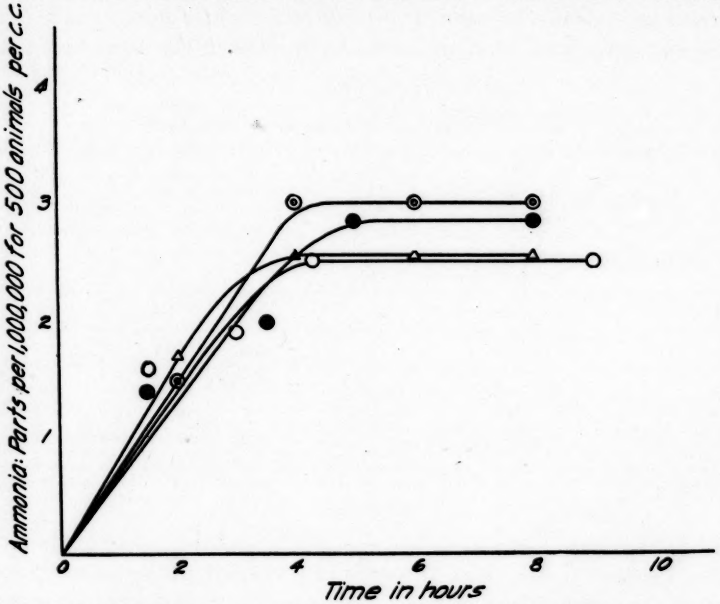

FIG. 3.—*Spirostomum:* concentration of urea in surrounding medium at successive periods of time as indicated by the amount of ammonia produced by hydrolysis. Amount of ammonia produced by hydrolysis at successive periods of time. △, Exp. 1; ⊙, Exp. 2; ●, Exp. 3; ○, Exp. 4.

to methods previously described. In two of the tests only a few *Paramecia* were added as food to the fluid containing the *Didinia.* From these experiments were obtained curves 2 and 3 (fig. 4). In the other two enough *Paramecia* were added to provide sufficient food for the duration of the experiments. These *Paramecia* had been starved for a period of 12 hours or longer, so the amount of nitrogenous substances excreted by them was insignificant. The results of these four experiments are presented in Table IV and in Figure 4.

By comparing curves 1 and 4 with curves 2 and 3 in Figure 4, it will at once be seen that the rates of excretion of ammonia under the two sets of conditions differed greatly. In Experiments 2 and 3 there were not enough *Paramecia* to maintain a supply of food throughout the tests. After the food which was present had been exhausted, the rate of excretion decreased markedly. The curves for these experiments are similar to those obtained for *Paramecium* and *Spirostomum*, and they are to be interpreted in the same manner.

TABLE IV

RATE OF EXCRETION OF AMMONIA BY *Didinium**

Exp. No.	No. of Animals per Cc.	Food Supply	Parts of Ammonia by Weight per 1,000,000 for 200 Animals per Cc. after—								
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.	9 Hr.
1	200	suf.	0.85	1.70		3.10			5.50		7.85
2	312	insf.	0.80	1.45		1.88		2.30			
3	250	insf.		1.18		2.00		2.00			
4	160	suf.	0.80	1.55		2.90		4.60			

* The amount of ammonia excreted by 200 animals per cubic centimeter of culture solution in 1–9 hours is given in parts by weight per 1,000,000 of solution. The abbreviations "suf." and "insf." denote respectively sufficient and insufficient food. Even in those experiments marked "insf." there were a few *Paramecia* present. Note that the concentration of ammonia in the culture containing much food increases from the beginning to the end of the experiment but that in the culture with little food it increases very little after 2 hours.

In Experiments 1 and 4, however, the food supply was adequate for the duration of the tests. Here the rate of excretion remained constant throughout the period of the experiments, and did not decrease as in Experiments 2 and 3. It is concluded from this direct comparison of the effects of starvation and satiety that the rate of excretion depends upon the presence or absence of a sufficient supply of food just as it does in man.

III. METHOD OF EXCRETION OF NITROGENOUS END-PRODUCTS OF METABOLISM IN PROTOZOA

It is often asserted that the nitrogenous end-products of metabolism in Protozoa are eliminated by the contractile vacuole. There is, however, considerable evidence which indicates that the vacuole is primarily concerned with the removal of excess water which ac-

cumulates in the cell. A summary of the evidence favoring each of these views follows:

Stein (1878) probably was the first to maintain that the function of the contractile vacuole is excretory. Stein and Schmidt (see Kent, 1880, p. 69) make the following statement: "The functions

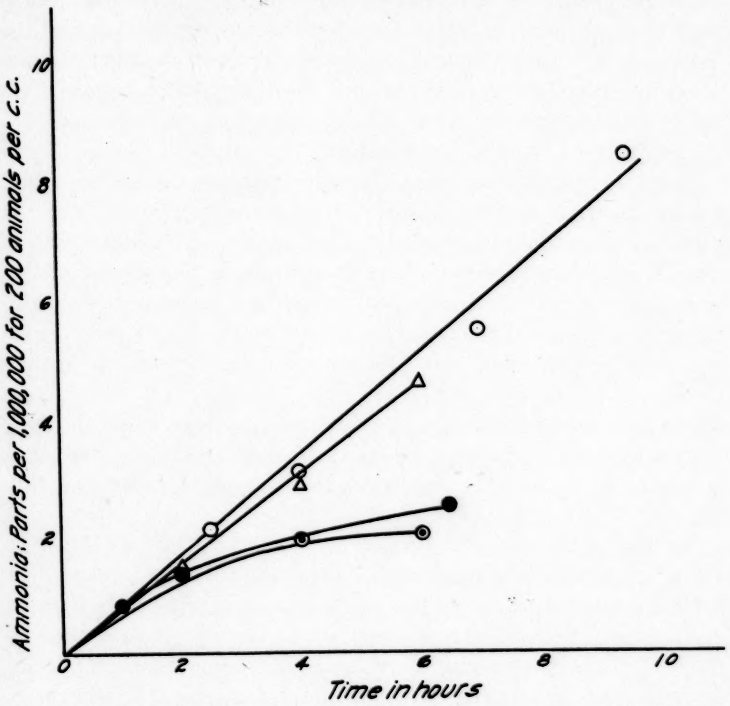

FIG. 4.—*Didinium:* amount of ammonia present at successive periods of time. ○, Exp. 1; ●, Exp. 2; ⊙, Exp. 3; △, Exp. 4.

discharged by the contractile vacuole are excretory, and correspond most nearly to those of the renal organs of the higher animals." Among others who have supported the excretory theory are Pritchard (1861), Griffiths (1888), Khainsky (1910), Woodruff (1911), Minchin (1912), and Howland (1924). The evidence given by Griffiths and contradicted by Howland has been presented above.

Khainsky (1910) found that an increase in temperature, within certain limits, is accompanied by a corresponding increase in the pulsation-rate. This was interpreted as indicating a higher rate of metabolism, the more rapid pulsation-rate being necessary to remove the increased amount of waste-products.

Experiments by Nowikoff (1908), Shumway (1917), the Riddle and Torrey (1923), in which the effects of thyroid feeding and the response of *Paramecia* to thyroxin were observed, offer further though indirect evidence in favor of the theory of excretion. Two definite effects were noted: a tendency toward the formation of extra vacuoles, and a marked acceleration in the rate of pulsation.

Flather (1919) found that adrenalin, pituitrin, and pineal gland extract produce similar results—a pronounced acceleration in the pulsation-rate, and a dilatation of the vacuoles. It is interesting to note in this connection that these drugs produce diuresis and dilatation in the vertebrate kidney, accompanying a general rise in blood-pressure; and that the diuresis and dilatation last longer than the increased blood-pressure, indicating a specific effect on the kidney itself.

In his discussion of the functions and homologies of the contractile vacuole in plants and animals Hartog (1888) suggested that it may be an organelle for regulating the hydrostatic pressure within the cell. This suggestion is based on the fact that in a system, such as a living cell presents, in which two fluids of different densities are separated by a semipermeable membrane, the less dense fluid is expected to pass through the membrane into the more dense fluid until a state of equilibrium is established. In the system formed by the cell, the endoplasm, the cell membrane, and the culture fluid represent respectively the more dense fluid, the semipermeable membrane, and the less dense fluid. If this obtains, endosmosis would be expected to take place, necessitating some form of mechanism to prevent excessive dilution of the endoplasm, and a hydrostatic pressure within the cell greater than the cell-membrane can withstand.

Zülzer (1910) found in experiments on *Amoeba verrucosa* that the pulsation-rate of the vacuole decreases with an increase in salt content of the culture-medium, and that concentrations of $1\frac{1}{2}$–$2\frac{1}{2}$ per cent of salt cause the vacuole to cease pulsating, but that upon res-

toration of the animals to fresh water pulsation is resumed. This seems to indicate that at these concentrations of salt the exosmotic effect exerted by the culture medium is equal to or greater than the endosmotic effect exerted by the endoplasm. If this is true, there is no excess water within the cell to be removed, and the vacuole is functionless. Whether or not the cessation of pulsation in Zülzer's experiments was due to the isotonicity of the culture-medium with the endoplasm or to pathological conditions induced by the salt has not been ascertained.

Stempell (1914) constructed a mechanical system which shows clearly that osmosis can be made the causal agent for producing intermittent discharge of a fluid from a system. Doubtless this system was not intended to be compared with the contractile vacuole in any respect other than the pulsating effect.

The relation found by Khainsky (1910) between increases in temperature and increases in pulsation-frequency can be explained on the basis of the hydrostatic theory as follows. Van Rysselberghe (1902) found that the permeability of living membranes increases with an increase in temperature. If this is true, then an increase in temperature would necessitate the removal of a larger amount of water which passes through the membrane because of its increased permeability. If the function of the vacuole is the removal of the excess water, then an increase in temperature would necessitate a corresponding increase in pulsation-frequency. The observed increase in rate of pulsation therefore is not necessarily due to an increased rate of metabolism as Khainsky maintains.

In a previous paper the author (1927) presented further evidence bearing on the question as to whether or not the function of the contractile vacuole is primarily that of an excretory organelle. It was shown in this paper that urea cannot be detected in the fluid of the vacuoles of *Paramecium* by means of the xanthydrol precipitation test described by Fosse (1913). The xanthydrol reagent, which is sensitive to one part of urea in 12,000 by weight when used in this manner, was injected into the vacuole with the aid of suitable microinjection apparatus. No trace of the characteristic needle-like crystals of di-xanthyl urea, which are precipitated by xanthydrol in the presence of urea, was found either in that part of the organism in

which the contractile vacuole is usually located or in the liquid surrounding the organism. It was consequently concluded that either urea is not present in the fluid of the vacuole or that its concentration is too low to be detected with the reagent used.

Further evidence bearing on the questions under consideration was obtained in experiments described below. These experiments consisted of removing the fluid from the vacuole and subjecting it to microchemical analysis.

MATERIAL AND METHODS

In these experiments *Spirostomum* was used. The organisms were washed free from culture fluid as previously described to prevent the possible introduction of error from contamination of the fluid of the vacuole by substances dissolved in the culture fluid.

The apparatus used for the removal of the contents of the contractile vacuole consisted of a Taylor (1925) pipette used in connection with a Chambers (1922) micromanipulator. The main body of the pipette was rigidly mounted on the table to the left of the micromanipulator and was connected to the pipette tip, which was mounted on the movable arm of the manipulator, by a glass tube approximately 40 cm. in length. This glass tube, having a diameter of 4 mm., was sufficiently flexible to permit a wide enough range of motion for the capillary tip. It is especially advantageous to have the main body of the pipette mounted separately and some distance removed from the capillary tip. This prevents to a considerable extent the transmission of vibrations from the pipette to the tip during adjustment and manipulation of the former. The organism was mounted on the lower surface of a cover glass. This cover glass formed the top of a rectangular glass chamber, the front of which was left open to allow the pipette entrance. The tip of the pipette was bent up at a right angle to facilitate its introduction into the body of the organism suspended on the lower surface of the cover glass.

By the use of this apparatus, fluid was removed from the vacuole and injected into a drop of a solution of urease hanging from the lower surface of another cover glass; then, after a short time, generally about 2 minutes, a drop of Nessler's reagent was added to the

drop of urease solution to ascertain whether or not ammonia was produced after the treatment of the contents of the vacuole with the enzyme.

RESULTS

Four experiments with *Spirostomum* such as described above were made. In two of these the contents of a single vacuole were removed; in the others the contents of two were removed. Upon the addition of Nessler's reagent the characteristic brown color produced by this reagent in the presence of ammonia was plainly visible under the microscope in all four experiments. In those in which the contents of two vacuoles were used the color developed was considerably deeper than in those in which the contents from only one were used. No indication of ammonia was obtained in other experiments in which the enzyme was not used. Nor did Nesslerization of a drop of urease solution alone show any trace of ammonia. The brown color obtained when the contents of the vacuole were added to the urease solution and then Nesslerized must therefore have arisen from ammonia produced by hydrolysis of urea dissolved in the fluid of the vacuole. It may consequently be concluded that urea is excreted by the contractile vacuole in *Spirostomum*. It will be recalled that the results obtained in experiments described in a preceding paper (Weatherby, 1927) indicate that this does not obtain in *Paramecium*. However, upon purely theoretical considerations, one would anticipate the fluid of the vacuole to contain dissolved in it at least a part of all soluble substances in the body. The fact, therefore, that urea was not found in *Paramecium* does not indicate that none was present; it merely indicates that the reagent used was not sensitive enough to give positive test at the concentration which obtained.

The question now arises as to whether the urea excreted by the contractile vacuole is sufficient in quantity to indicate that it is an excretory organelle. The depth of color developed in the tests for ammonia in the experiments on *Spirostomum* was, at most, comparable to that produced in a solution of ammonia containing one part by weight per 500,000. However, the fluid of the vacuole was diluted to from three to five times the original volume when added to the urease solution; this gives an original concentration of ap-

proximately one part by weight per 100,000 parts of fluid. Assuming that this was the concentration, what portion of the urea excreted by this form passes out through the contractile vacuole? As previously stated, the quantity of urea excreted by *Spirostomum* is approximately 1.25 parts by weight per 1,000,000 parts of culture fluid per hour for 500 animals per cubic centimeter. By ascertaining the rate of pulsation of the vacuole, and the volume of the vacuole, the quantity of fluid evacuated by a given number of organisms in a given length of time was computed. From this it was found that the concentration of urea in the fluid of the vacuole would be of the order of one part by weight per 1,000 parts of fluid, if all the urea excreted is eliminated through the vacuole. If the fluid of the vacuole, then, contains only one part of urea by weight per 100,000 parts of fluid, it is evident that not more than 1 per cent of the urea excreted passes through the vacuole.

On the basis of these considerations one is justified in concluding that the vacuole is not primarily an organelle of excretion as the term is usually defined. Moreover, the physical nature of the organism would lead one to predict that crystalloid substances such as urea, which are dissolved in the body fluid, readily pass directly to the exterior by dialysis. In all probability, then, this is the mechanism for the elimination of nitrogenous end-products of metabolism.

SUMMARY

1. The bulk of the nitrogen excreted by *Paramecium* and *Spirostomum* is in the form of urea. No excretion of ammonia, uric acid, creatin, or creatinin was found. The bulk of the nitrogen excreted by *Didinium* is in the form of ammonia. In experiments lasting for 24 hours or more a trace of uric acid was sometimes found, but no urea, creatin, or creatinin.

2. In *Paramecium* the initial rate of excretion of urea is approximately 1.40 parts by weight per 1,000,000 per hour for 2,500 animals per cubic centimeter.

3. In *Spirostomum* the initial rate of excretion of urea is approximately 1.30 parts by weight per 1,000,000 per hour for 500 animals per cubic centimeter.

4. In *Didinium* the rate of excretion of ammonia is approximately 0.80 parts by weight per 1,000,000 per hour for 200 animals per cubic centimeter. The rate of excretion of uric acid in this form is not sufficient to permit measurements with any considerable degree of accuracy.

5. Substances frequently used in the preparation of culture media—hay, wheat, barley, rye, rolled oats, malted milk, beef extract, blood fibrin, and blood albumen—contain considerable quantities of uric acid. This probably accounts for the fact that Howland found uric acid in cultures of Protozoa.

6. Methods for quantitative estimation of the amount of urea in the fluid of the contractile vacuole were developed. In *Spirostomum* this was found to be approximately one part by weight per 1,000,000.

7. The amount of urea which should be in the fluid of the vacuole, if all the urea excreted by this form is eliminated through the vacuole, is approximately one part by weight per 1,000, or about 100 times the amount which was actually found. In *Spirostomum*, then, the contractile vacuole does not function primarily as an excretory organelle, as the term is generally used.

8. That urea was not detected in the fluid of the vacuole in *Paramecium* in previous experiments was no doubt due to the fact that the reagent used was not sensitive enough to give a positive indication at the dilution which actually obtains. The reagent in these experiments was sensitive to only one part of urea by weight per 12,000. The actual concentration of urea in the fluid of the vacuole in *Paramecium*, if *Paramecium* may be compared to *Spirostomum* in this respect, is more nearly of the order of one-tenth that amount.

9. The physical structure of a protozoan such as *Paramecium* or *Spirostomum* indicates that at least part of all soluble substances contained within the body pass to the exterior through the cell membrane by the process of dialysis. If this obtains, a part of the urea, which is an excretory product in these forms, is excreted through the vacuole; but the greater part passes by dialysis directly to the exterior through the cell membrane.

LITERATURE CITED

ADOLPH, E. F. 1922. The physiological action of excretory products. Anat. Rec., XXIV, 396.

CHAMBERS, R. 1922. New apparatus for the dissection and injection of living cells. *Ibid.*, pp. 1–19.

FLATHER, M. D. 1919. The influence of the glandular extracts upon the contractile vacuoles of *Paramecium caudatum*. Biol. Bull., XXXVII, 22–39.

FOSSE, R. 1913. Sur l'identification de l'urée et sa précipitation de solution extrêmement diluées. Comp. Rend., CXXXVII, 948–57.

GRIFFITHS, A. B. 1888. A Method of demonstrating the presence of uric acid in the contractile vacuoles of some of the lower organisms. Proc. Royal Soc. Edin., XVI, 131–35.

HARTOG, M. M. 1888. Preliminary notes on the functions and homologies of the contractile vacuole in plants and animals Rep. British Assoc. Adv. Sci., pp. 714–16.

HOWLAND, RUTH B. 1924. On excretion of nitrogenous waste as a function of the contractile vacuole. Jour. Exp. Zoöl., XL, 231–50.

KENT, W. S. 1880. A manual of the infusoria. London. Pp. 913.

KHAINSKY, A. 1910. Zur Morphologie und Physiologie einiger Infusorien (*Paramecium caudatum*) auf Grund einer neuen histologischen Methode. Arch. f. Prot., XVI, 1–60.

MINCHIN, E. A. 1912. An introduction to the study of the protozoa. London. Pp. 504.

MORSE, W. 1927. Applied biochemistry. Philadelphia and London. Pp. 988.

NOWIKOFF, M. 1908. Über die Wirkung des Schilddrüsen Extrakts und einiger anderer Organstoffe auf Ciliaten. Arch f. Prot., XI–XII, 309–26.

PRITCHARD, A. 1861. A history of the infusoria. London. Pp. 986.

RIDDLE, M. C., and TORREY, H. B. 1923. The physiological response of *Paramecium* to thyroxin. Anat. Rec., XXIV, 396.

SHUMWAY, WALDO. 1917. The Effect of a thyroid diet on *Paramecium*. Jour. Exp. Zoöl., XXII, 529–62.

STEIN, F. 1878. Der Organismus der Infusionsthiere nach eigenen Forschungen in systematischer Reihenfolge bearbeitet. Leipzig. Div. 3, p. 154.

STEMPELL, W. 1914. Über die Funktion der pulsierenden Vacuole und einen Apparat zur Demonstration derselben. Zool. Jahrb., Abt. f. Allg. Zool. und Physiol. d. Thiere, XXXIV, 437–78.

TAYLOR, C. V. 1925. Improved micromanipulation apparatus. Univ. Cal. Pub. Zoöl., XXVI, 443–54.

VAN RYSSELBERGHE, Fr. 1902. Influence de la température sur la permeabilité du protoplasme vivant pour l'eau et les substances dissoutes, Rec. Inst. Bot. Brux., V, 209–49.

ZÜLZER, MARGARET. 1910. Über den Einfluss des Meerwassers auf die pulsierende Vacuole. Arch. f. Entw. d. Org., XXIX, 632–40.

THE PHYSIOLOGY OF THE CRUSTACEAN HEART. II. THE EFFECT OF LITHIUM, AMMONIUM, STRONTIUM, AND BARIUM IONS UPON THE HEART RHYTHM OF THE CRAYFISH (CAMBARUS CLARKII)

(Seven figures)

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INTRODUCTION

THE present investigation is a continuation of an effort to determine the effect of various ions upon the heart rhythm of the crayfish. In a previous paper (1928) the author reported the results of a series of studies on the effect of Na, K, Ca, Mg, and hydrogen ions upon the heart rhythm of this species. In the foregoing work a method was devised whereby the pulsations of the intact heart of the crayfish could be recorded and studied graphically, while being perfused with various ionic solutions. Furthermore, various concentrations of NaCl, KCl, and $CaCl_2$ were tested together in a perfusing bath in an attempt to find a solution which would maintain normal heart rhythm. A solution referred to frequently as a "normal perfusing solution" and containing 1 per cent NaCl plus 0.024 per cent $CaCl_2$ plus 0.028 per cent KCl, fulfilled this purpose and kept the heart beating normally for several hours when exposed and perfused at a temperature of 25° C. This solution was employed as a control bath and as a basis for observing the effect of solutions containing different concentrations of the various ions tested. The experiments reported in the present paper are of a similar nature but employ other ions found in the same periodic classification of metals, namely, Li and NH_4 in the alkali group and Sr and Ba in the alkali-earth group. Although these particular ions are probably not concerned in the normal physiological activity of the crayfish heart, they may through substitution throw some light on the nature of the action of normal blood ions. To quote Mines (1911), "the most promising way of attacking the problem of how this or that ion serves its peculiar rôle in living tissue is to discover

how far it can be replaced by other ions and what are the physical and chemical relations of those found to be effective substitutes."

The observations hitherto recorded dealing with the effect of ions upon the hearts of various crustaceans have been performed almost exclusively with Na, Ca, K, and Mg ions. Moreover, the bulk of the investigations have centered entirely around larger marine forms, notably lobsters and crabs. Concerning the work on these latter forms in which the ions of Li, Sr, Ba, and NH_4 have been employed, the works of Hogben (1925) and Zoond and Slome (1928) are of interest. The former made an extensive study of the action of Li on the heart of the lobster. He noted that when LiCl was substituted for NaCl in molar equivalent amounts in the normal media, the amplitude of the beats was progressively diminished by a decrease in systolic tone while the frequency was accelerated and the heart slowly passed into diastolic stoppage. Furthermore, the attempt to substitute Ba or Sr for Ca in the normal perfusing media proved unsuccessful. Zoond and Slome (1928) working with *Palinurus* found that considerable amounts of Sr were necessary in the perfusing solution to evoke any noticeable response in the heart. They noted, however, that when large amounts were added, there was an increase in tone. Very small amounts of Ba produced an increase in tone, accompanied by reduced frequency and ultimately a condition of contracture.

In view of the fact that practically all work on the effect of Li, NH_4, Sr, and Ba on crustacean heart rhythm has been carried out with marine forms, it seemed worth while that a study be made on a fresh-water form. The crayfish provides very favorable material for such an investigation and was therefore selected. The purpose of the present work was twofold: first, to determine and record, individually, the effect of Li, NH_4, Ba, and Sr ions upon the heart rhythm of the crayfish; and, second, to attempt the replacement of any one of these ions in the normal perfusing solution with Li, Ba, or Sr.

The writer wishes to take this opportunity to express his gratitude to Dr. O. M. Helff, not only for suggesting the problem, but also for his kindly interest and criticism throughout the course of the investigation.

MATERIAL AND METHODS

The species of crayfish used in this investigation was *Cambarus clarkii*, a shallow-water species native to the swamps and fresh-water marshes of Louisiana. The animals were collected and shipped from Louisiana and upon arrival were placed in a large tank of running water for several days before being used for experimental work. The purpose of this precaution was to prevent erratic results, due to possible disturbances during shipping or to changes in the environment. The individuals selected for use ranged from 3 to 5 inches in length and were normal animals in every respect.

The general technique employed in obtaining graphic records of the effect of various ions on the heart rhythm has been previously fully described (Lindeman, 1928). Briefly, however, it consisted of placing the animal in a specially constructed clamp which held it securely in a horizontal position and prevented the appendages from interfering with the accuracy of the recording apparatus. A portion of the carapace, body wall, and pericardium was dissected away, thus exposing the heart. The heart was then attached to a heart lever by means of a very fine wire and steel hook. The recording apparatus consisted of the usual set-up including a kymograph, heart lever, and signal magnet, the latter operated by means of an electrical-clock timing apparatus. The pulsations were recorded upon a slowly moving drum.

To insure that all hearts were perfused at a constant rate for all solutions employed, a series of delivery siphons was arranged, one for each solution, whose rate of flow could be accurately regulated by means of screw clamps. The perfusing solutions were allowed to run into the pericardial sinus, from which point they were quickly perfused through the heart by means of the ostia. The temperature of the perfusing solutions was maintained at 25° C. and the pH between 6.9 and 7.1.

A solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$, previously reported by the author as adequate for maintaining normal heart rhythm in this species, was employed as a control bath and as a basis for observing the effect of the various ions. For convenience, this solution will be referred

to as the "normal perfusing solution" throughout the present paper and will be designated by *N* on all graphic records.

The experiments consisted in first perfusing the intact heart for several minutes with the normal perfusing solution. This removed most of the blood from the heart and pericardial sinus. Furthermore, this also served to eliminate those individuals which, because of imperfect operative technique, displayed an irregular heart rhythm. In such cases where the normal perfusing solution failed to restore the normal rhythm within 5 minutes, the animals were discarded and not used for further experimentation. When the heart rhythm continued normal, however, the normal perfusing solution was discontinued and the ionic solution to be tested applied immediately at the same rate, and the effect on heart rhythm recorded graphically.

RESULTS

LITHIUM

The effect of lithium upon the heart rhythm of the crayfish appears to be one of considerable complexity. Within certain limits LiCl and NaCl are quite analogous in their effects, at least on the species of crayfish employed. The author has shown (Lindeman, 1928), that 1 per cent NaCl solution at first produces a rapid decrease in tone and an increase in rate and amplitude. With continued perfusing, however, arrest is produced in about 30 minutes. LiCl in an equivalent concentration causes an increase in tone, followed shortly by arrest. However, upon the application of a 0.5 per cent LiCl solution to the intact heart, a decrease in tone immediately occurs, coupled with an increase in rate and amplitude for a short time (Fig. 1*A*). With continued perfusing, the rate and amplitude gradually decreased, with ultimate arrest in about 25 minutes. Figure 1*B* represents the type of pulsations obtained at the close of a 15-minute application of the 0.5 per cent LiCl solution.

Because of the apparent similarity of a pure solution of 0.5 per cent LiCl and a 1 per cent NaCl solution in their action upon heart rhythm, an attempt was made partially to replace the NaCl in the normal perfusing solution with LiCl. If the intact heart of the crayfish is perfused with a solution containing 0.7 per cent NaCl plus 0.3 per cent LiCl plus 0.028 per cent KCl plus 0.024 per cent CaCl.,

it will continue to beat quite normally for several hours. Figure 2*A* is a record of a heart perfused with such a solution. Figure 2*B* represents the type of pulsations obtained at the close of a 1-hour application. At this time, it will be noted, the pulsations are more frequent and appear slightly stronger than normal.

An attempt to completely replace the NaCl with LiCl proved less successful. Upon substituting 0.5 per cent LiCl for the 1 per

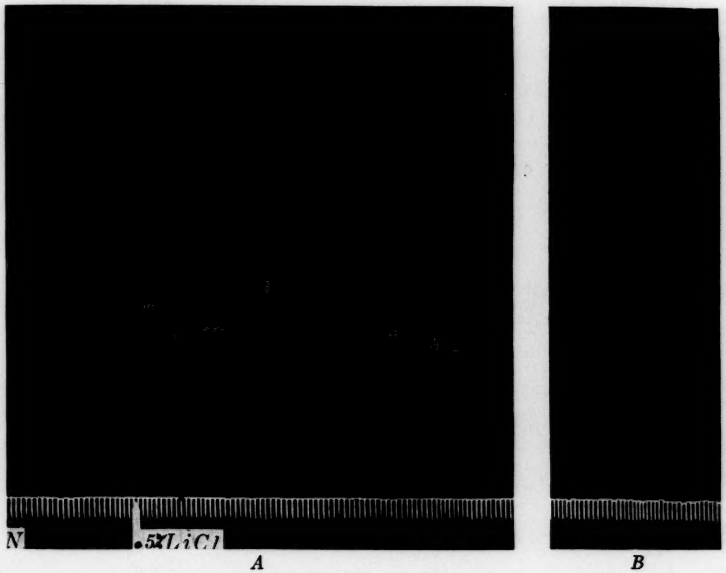

FIG. 1

cent NaCl in the normal perfusing solution, a slight increase in tone follows, coupled with a slight increase in rate and amplitude of the beats. However, with continued perfusing, the amplitude slowly decreases, resulting ultimately in stoppage after a period of about 45 minutes. The substitution of an equivalent concentration of LiCl for the 1 per cent NaCl in the normal perfusing solution gave similar results but with a greater degree of intensity, the arrest developing much earlier. The results obtained by the writer for crayfish compare quite closely with those obtained by Hogben (1925) for *Homa-*

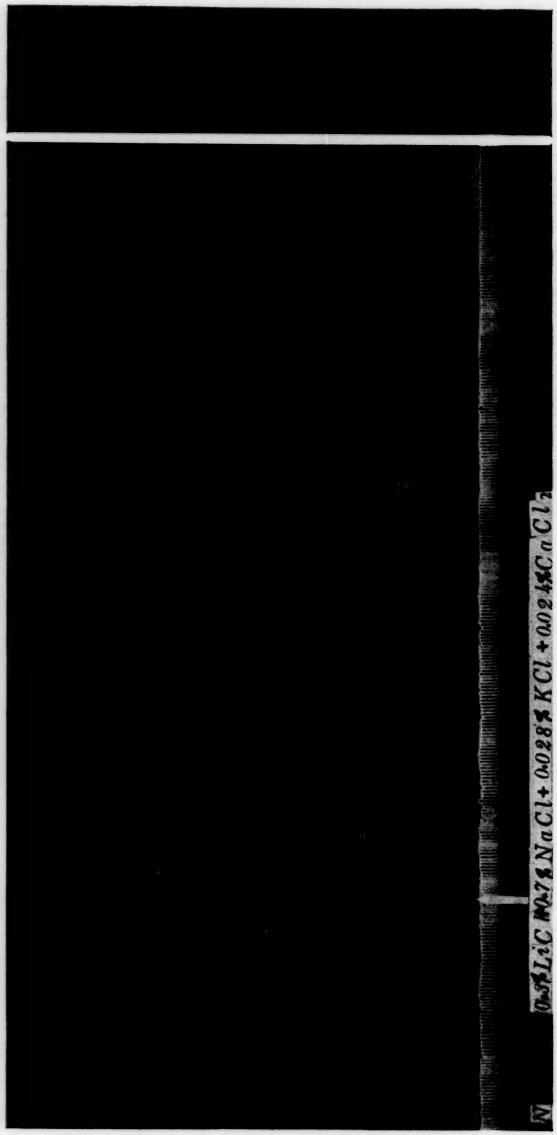

FIG. 2

rus, demonstrating that Li is unable completely to replace Na in a perfusion medium.

That Li is capable of partially replacing Na in the normal perfusing solution appears quite evident. The action of LiCl upon the cardiac mechanism is undoubtedly more violent than NaCl. The fact that a 0.5 per cent solution of LiCl produces almost identically the same effect upon heart rhythm as does a 1 per cent NaCl solution suggests, however, that the heart of the crayfish is more sensitive to the action of Li than to equivalent concentrations of Na. The exact nature of the influence of lithium, however, is quite problematic.

STRONTIUM AND BARIUM

It is generally recognized that strontium resembles calcium in its physiological action upon muscular tissue and is capable, within certain limits, of replacing Ca more or less completely in the physiological process of contraction. The power of Ba to replace Ca in this respect is undoubtedly very much more limited, although not entirely lacking. In this connection Mines (1911) found that Ca, Sr, and Ba show a gradation of physiological activity in relation to certain neuromuscular mechanisms in the frog. In respect to cardiac muscle, Mines recognized a gradation in their influence upon tone, and in their capacity to facilitate the transmission of excitation from nerve into muscle. Ringer (1886) demonstrated a very perfect substitution of Ca by Sr as far as maintaining the heart beat of the frog was concerned.

The effect of Sr upon the heart of the crayfish was found to be quite analogous to that of Ca, already described by the author (Lindeman, 1928). Upon the application of a 0.028 per cent $SrCl_2$ solution to the intact heart, a sharp increase in tone immediately follows, coupled with a slight irregularity in the rhythm (Fig. 3). With continued perfusing, the rhythm is slightly depressed, with ultimate arrest in less than 5 minutes. Subsequent application of the normal perfusing solution, shortly before arrest, results in complete recovery of the normal rhythm (Fig. 3).

In order to test the further relationship of Ca and Sr in regard to their effect upon heart rhythm, a 0.020 per cent solution of $SrCl_2$ was substituted for the 0.024 per cent $CaCl_2$ in the normal perfusing

solution. Upon the application of such a solution, containing 1 per cent NaCl plus 0.020 per cent $SrCl_2$ plus 0.028 per cent KCl, a slight increase in tone generally occurs, coupled with a slight decrease in the rate of the pulsations (Fig. 4*A*). With continued perfusing, this rhythm will continue in some cases for as long as 5 hours. Figure 4*B* represents the type of pulsations obtained at the close of a 3-hour application. It will be noted that the contractions are strong and give evidence of continuing their normal rhythm for some time.

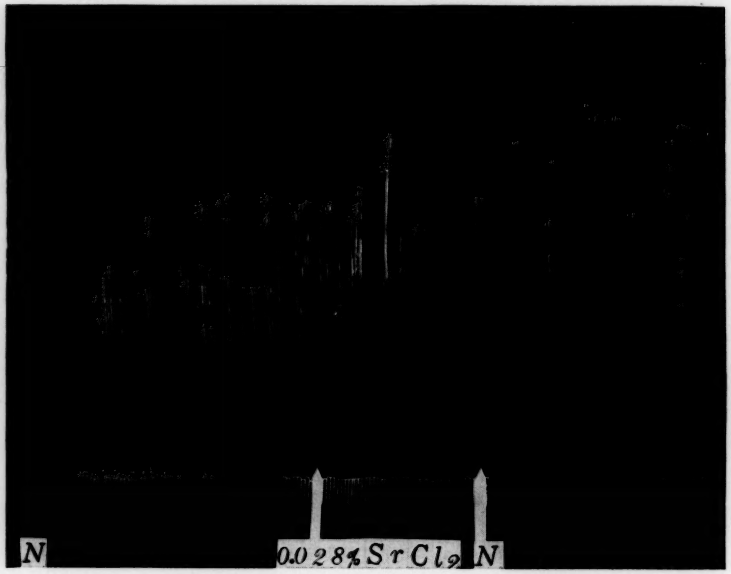

FIG. 3

In striking contrast to these results obtained upon the crayfish, Hogben (1925) was unable to substitute Sr for Ca in relation to hearts of both *Maia* and *Homarus*. Zoond and Slome (1928), however, find that while the heart of *Palinurus* is very insensitive to small amounts of Sr, an excess produces the same effect upon heart rhythm of this form as is true of a Ca excess; notably an increase in tone.

The action of Ba on the crayfish heart is entirely different from

that of Sr and Ca. The power of Ba to replace Ca is entirely lacking in this regard. $BaCl_2$ always produces an increased tonicity of the heart which leads ultimately to systolic arrest. Upon the application of a 0.024 per cent $BaCl_2$ solution to the intact heart of the crayfish an increase in tone occurs, followed by an irregularity in the rhythm (Fig. 5). Systolic arrest is generally obtained in less than 2 minutes. Subsequent application of a normal perfusing solution fails to restore

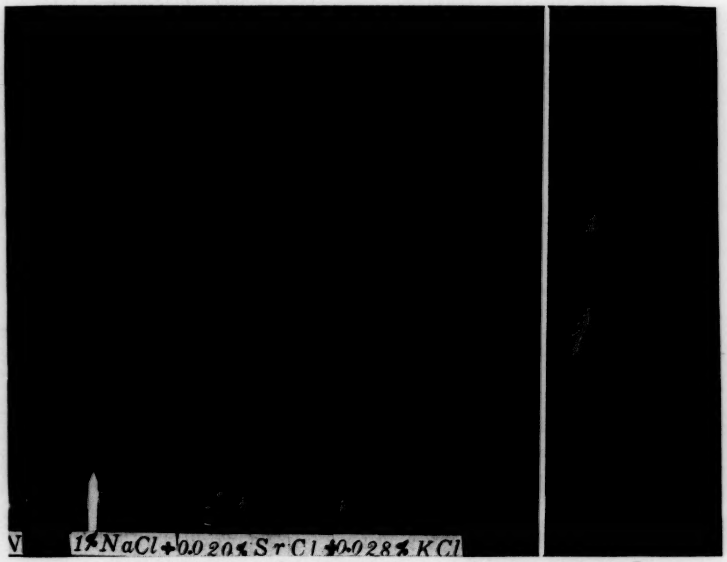

FIG. 4

the pulsations. When a 0.020 per cent $BaCl_2$ solution is substituted for the 0.024 per cent $CaCl_2$ in the normal perfusing solution and perfused through the heart, a slight increase in tone occurs. With continued perfusing, there is a gradual increase in tone and a decrease in amplitude, resulting in arrest in a few minutes. Figure 6 represents the type of pulsations obtained with such a perfusing solution. If the $BaCl_2$ is decreased to 0.010 per cent in the normal perfusing solution and perfused through a heart which had been beating normally with the normal perfusing solution, similar results

are obtained but with less intensity. There occurs a slight decrease in rate and amplitude and a gradual increase in tone. The strength of the pulsations gradually becomes weaker and less regular, with systolic arrest generally intervening in less than 10 minutes. In no case was it possible to restore the normal rhythm when the heart

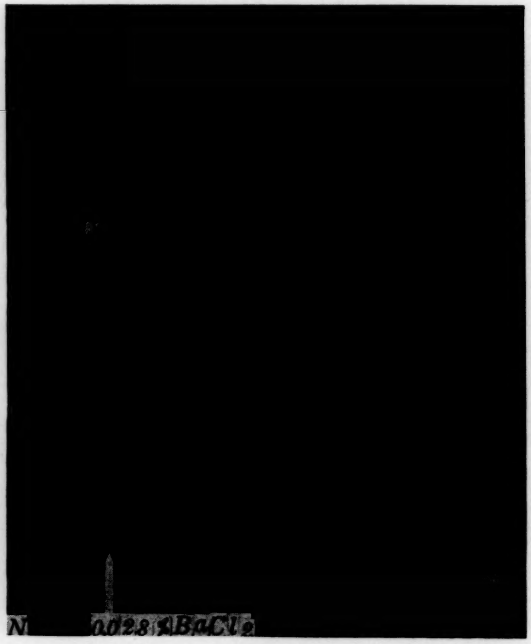

FIG. 5

was again perfused with the normal perfusing solution. Somewhat corresponding results have been shown by Hogben (1925) for *Maia* and *Homarus* and by Zoond and Slome (1928) for *Palinurus* in this respect. These workers demonstrated that the presence of Ba in very weak concentrations was sufficient to bring about stoppage in systole within a few minutes. According to the former worker, however, the effect was not particularly a toxic one, inasmuch as the heart of *Maia* in particular gradually recovered a comparatively

normal rhythm when restored to its normal perfusing medium. In the case of *Homarus*, complete recovery was not obtained.

The power of Ba to replace Ca in the mechanism of the crayfish heart rhythm has been shown to be very limited, if not entirely lacking. The fact that Ba in very weak concentrations is capable of producing systolic stoppage from which no restoration of pulsations can be obtained, suggests very strongly that Ba is injurious

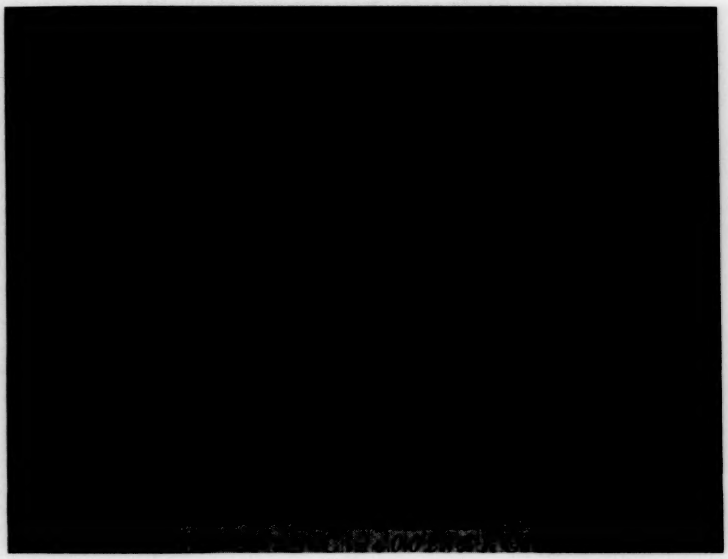

FIG. 6

to the tissue. Mines (1911) suggests that Ba forms an insoluble compound with some cell constituent. From the results obtained upon the crayfish this view is given further support. Moreover, such an assumption would also provide a logical explanation for the irreversible reaction obtained in the case of crayfish heart rhythm.

AMMONIUM

The effect of ammonium on the mechanism of the crayfish heart appears to be one of depression. Low concentrations of ammonium chloride added to the normal perfusion solution produced a decrease

in the strength and frequency of the pulsations. A solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$ plus 0.040 per cent NH_4Cl, when applied to the normally pulsating heart, produced an increase in tone and a decrease in the rate and amplitude of the beats. With continued perfusing, systolic

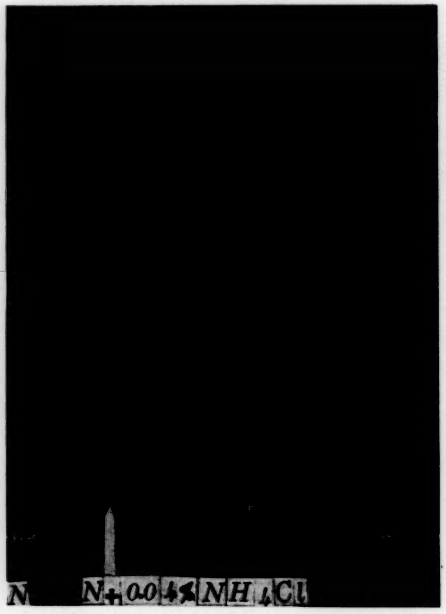

FIG. 7

arrest generally resulted in a few minutes (Fig. 7). Ionic concentrations of less than 0.010 per cent NH_4Cl added to the normal perfusing solution resulted in arrest in from 10 to 20 minutes, depending upon the concentration. In only a few cases were the pulsations restored upon the application of the normal perfusing solution.

DISCUSSION

From the above-described observations it is clear that various ions normally found in the blood, such as Na and Ca, can be partially

or entirely replaced by closely related ions in the mechanism of the crayfish heart rhythm. The power of Sr to replace Ca in this respect is the most outstanding example. This is not surprising, considering their similar chemical properties which place them in the same periodic classification of metals. However, it is interesting to note the wide variation in the response of various invertebrate hearts to the substitution of Sr for Ca in the perfusion medium. Mines (1911) noted that substitution of Sr for Ca in equivalent concentration in the perfusion media caused a rise in tone and a decrease in the frequency of the heart beat of *Pecten*. He did not, however, make an extensive study of the relationship of Ca and Sr but suggested that it might be possible to get a better effect by the substitution of smaller concentrations of Sr for a certain concentration of Ca instead of using chemically equivalent concentrations. Hogben (1925) and Zoond and Slome (1928), already referred to, working upon various marine crustaceans and mollusks, found that relatively high concentrations of Sr were necessary in the perfusion media before any noticeable response could be obtained. Moreover, substitution of Sr for Ca was unsuccessful.

The fact that Sr is capable of replacing Ca in the mechanism of frog heart rhythm has been clearly demonstrated by Ringer (1886), Kirsch (1927), Hirsch and Oppenheimer (1925), and others. Ringer in particular was able to demonstrate quite clearly a perfect substitution of Sr for Ca in Ringer's solution.

The findings in the present paper show quite conclusively that Sr and Ca produce similar effects upon crayfish heart rhythm. Moreover, Sr in concentrations slightly less than equivalent concentration of Ca is capable of completely replacing the Ca in the normal perfusing solution. It is concluded, therefore, that Ca and Sr are somewhat analogous in their effects upon the crayfish heart.

The power of lithium to replace sodium in this respect is less striking. Although certain of their chemical properties are quite analogous, they nevertheless produce peculiarities in the normal heart rhythm which are not common to both ions. The most striking difference to be noted is in the concentration of the two ions at which similar effects upon heart rhythm were produced. It will be recalled that a 0.5 per cent LiCl solution produced an effect similar

to that typical of a 1 per cent NaCl solution. However, when 0.5 per cent LiCl was substituted for the 1 per cent NaCl in the normal perfusing solution, a slight increase in tone occurred, with a decrease in amplitude and arrest in about 45 minutes. From the peculiarities noted in the rhythm it is clearly seen that lithium lacked some of the fundamental properties necessary for maintaining normal heart rhythm in the crayfish and is therefore unlike sodium in this respect. It would appear probable that there is a profound difference between the mode of action of the lithium and sodium ions, although both produce a somewhat similar end-effect upon the heart rhythm in pure solutions. The type of graphic records obtained suggests that lithium does not have the properties necessary to antagonize the effect of the K ions present in the perfusing solution.

It is interesting to note that these observations confirm those of Hogben (1925) for *Homarus* in this respect. Duliere and Noyens (1927), however, report that for the frog's heart Na is largely replaceable by Li in the perfusion media. That Li is capable of partially replacing Na in the normal heart mechanism of the crayfish was demonstrated quite conclusively. Preliminary experiments had shown that a normal perfusing solution containing less than 1 per cent NaCl fails to maintain the normal heart rhythm. It will be recalled, however, that a solution containing 0.3 per cent LiCl plus 0.7 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$, when applied to the normally beating heart, produced very little change in the rhythm and maintained the normal heart beats for several hours. It would appear, therefore, that lithium is capable, to some extent, of partially replacing sodium in a normal perfusing solution.

In the case of Ba, no successful substitution of Ba for Ca in the normal perfusing solution could be obtained. The heart of the crayfish exhibits a remarkable sensitivity to the Ba ion even at very low concentration. Very low concentration applied to the normally beating heart leads to increased tone and occasional pauses in a systolic condition with ultimate systolic arrest from which no recovery can be obtained. From all appearances the nature of the action of Ba upon the heart seems to be one of producing rigor. As a matter of general interest it may be mentioned at this time that

after arrest of the heart pulsations with a solution containing Ba, very peculiar twitchings generally occurred for a time before complete arrest. The nature of these twitches did not appear to be the result of reduced frequency but rather of a lack of co-ordination between various parts of the heart itself. From all indications it would appear that Ba permanently injures crayfish heart tissue, although the exact nature of the injury is quite problematic.

SUMMARY AND CONCLUSION

1. A method, already described in an earlier paper, was employed whereby the pulsations of the intact crayfish heart could be recorded and studied graphically while being perfused with various ionic solutions.

2. A solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$ was employed as a normal bath for comparing the effect of various ionic solutions.

3. To determine the effect of the lithium ion upon heart rhythm, two perfusing solutions were employed, namely, one containing 1 per cent LiCl, and the second, 0.5 per cent LiCl. The 1 per cent solution produced greatly increased tone and arrest in a few minutes; the latter increased the rate and amplitude and decreased the tone, thereby producing an effect similar to a 1 per cent NaCl solution. It is concluded that Li and Na are, within certain limits, analogous in their effect upon crayfish heart rhythm.

4. In an attempt to determine to what extent lithium is capable of replacing sodium in the normal perfusing solution, three solutions were employed; one containing 1 per cent LiCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$, another containing 0.5 per cent LiCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$, and the third containing 0.3 per cent LiCl plus 0.7 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$. Both solutions in which Na was completely replaced increased the tone and decreased the amplitude of the beats. Arrest was obtained within 10 and 40 minutes, respectively. The third, in which Na was partially replaced by Li, maintained normal rhythm for several hours. It is concluded that Li is capable of partially replacing Na in the cardiac mechanism of the crayfish.

5. Similar experiments were performed with solutions containing $SrCl_2$. A pure solution of 0.028 per cent $SrCl_2$ produced increased tone and decreased rate, with arrest in about 5 minutes. Substitution of 0.020 per cent $SrCl_2$ for the 0.024 per cent $CaCl_2$ in the normal perfusing solution produced no noticeable change in normal heart rhythm for from 3 to 5 hours. In general it may be said that the Sr and Ca ions are analogous in their effect upon crayfish heart rhythm.

6. The presence of barium in a perfusing solution always produces an increased tonicity leading ultimately to systolic arrest. Perfusing with normal perfusing solution fails to restore the rhythm. It seems probable, therefore, that the Ba ion is injurious to cardiac muscle.

7. Normal perfusing solutions in which various concentrations of NH_4Cl have been added produce a depressor effect upon crayfish heart rhythm.

LITERATURE CITED

DULIERE, W., and NOYENS, A. K. 1927. Le lithium dans le liquide de perfusion du cœur de grenoville. Compt. rend. Soc. de biol., XCVII, 193.

HOGBEN, L. T. 1925. The action of electrolytes on invertebrate muscle. Quar. Jour. Exp. Physiol., XV, 263.

HIRSCH, S., and APPENHEIMER, A. 1925. Über die Wirkung von Strontium auf das Kalt- und Warmblüterherz. Arch. f. Expt. Path. u. Pharmak., CX, 89.

KIRSCH, B. 1927. Die Wirkung von $MgCl_2$, $SrCl_2$ und $BaCl_2$ auf die Herzreizbildung beim Kaltblüter. *Ibid.*, CXXIV, 210.

LINDEMAN, V. F. 1928. The effect of various ions upon the heart rhythm of the crayfish (*Cambarus clarkii*). Physiol. Zoöl., I, 576.

MINES, G. R. 1911. On the replacement of calcium in certain neuro-muscular mechanisms by allied substances. Jour. Physiol., XLII, 251.

———. 1912. On the relations to electrolytes of the hearts of different species of animals. *Ibid.*, XLIII, 467.

RINGER, S. 1886. Further experiments regarding the influence of small quantities of lime, potassium and other salts on muscular tissue. *Ibid.*, VII, 291.

ZOOND, A., and SLOME, D. 1928. The relation of electrolytes to the cardiac rhythm of *Palinurus* (*Jasus*) *Lalandii* and *Octopus* horridus. Brit. Jour. Exper. Biol., VI, 79.

EXPERIMENTAL STUDIES OF THE BIRD HYPOPHYSIS. I. EFFECTS OF HYPOPHYSECTOMY IN THE BROWN LEGHORN FOWL[1]

(Eighteen figures)

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INTRODUCTION

IN THE numerous experimental investigations into the functions of the pituitary gland within recent years, the birds have been neglected as subjects of experimental study. Fichera (1905) performed forty hypophysectomies on young chicks, and this is the most extensive study of the avian pituitary found in the literature. Fichera does not report exact age and breed of his birds, or give illustrations of his histological findings, though precautions were taken against infections and thorough autopsies were resorted to. Microscopic sections were emphasized as the criterion of extent of elimination of the anterior lobe. Fichera considered only four of his cases to be complete removals; and, as these survived, the author concluded that the gland is not indispensable to life. Immediate symptoms following its destruction the author concluded were due to the operative act, but a slight retardation of development of young chicks following loss of anterior lobe is reported. Although other endocrine organs were preserved, no changes were reported in them. An extension of these studies does not seem to have been made.

It is quite unnecessary to give a review of the extensive literature on the hypophysis in mammals and in Amphibia. On the question of its indispensability may be mentioned the work of Adler (1914), Smith (1916), Allen (1918), Hogben (1922), and others, in general agreement that in Amphibia the gland may be removed without causing the death of the animal. Among investigators on

[1] From the Whitman Laboratory of Experimental Zoölogy, University of Chicago. The expenses of this investigation were borne in large part by grants to this laboratory by the Sex Research Committee of the National Research Council.

mammals there is no general agreement. Excluding the earlier and probably less reliable results, we find Crowe, Cushing, and Homans (1910), Bell (1917), Dott (1923), and others expressing the view that the anterior lobe of the pituitary is essential in dogs. On the other hand, Aschner (1912), Brown (1922), and Dandy and Reichert (1925) claim complete removal of the pituitary of the dog without fatal results. In rats, P. E. Smith (1926) has removed the pituitary without causing the death of the animals. It appears that the explanation of these conflicting reports is probably to be found in differences of operative procedure, or else in physiological differences of the experimental animals used. However, the question of indispensability has become secondary to the study of pituitary excess or deficiency and, more recently, of the interglandular relationships of the endocrine system. Briefly, the literature may be summed up under the following heads:

1. *Growth effects.*—There is very general agreement that loss or deficiency of the anterior lobe secretion results in a retardation of growth or distinct dwarfing in mammals, birds, and Amphibia. Conversely, evidence points to renewed or excess growth following anterior hypophyseal administration. (Cf. Uhlenhuth [1920], who found salamander larvae outgrew controls when fed anterior lobe; Evans and Long [1921], Evans [1924], Evans and Simpson [1926], who show that injections of anterior lobe extract promote growth in the rat.)

2. *Relation to genital system.*—In mammals it seems established that lack or deficiency of the anterior-lobe hormone results in a failure of development of the genital system of the young animal, or its atrophy in the old (Crowe, Cushing, and Homans, Dott, Smith, etc.). That this is due to failure of the gonads themselves is indicated by the lack of effect of pituitary transplants on the genital system when the gonads have been previously removed (Smith and Engle, 1927). In the rat and mouse the opposite effect, namely, a stimulation of the gonads, occurs when the anterior lobe secretion is augmented by the daily introduction of anterior hypophyseal substance (Smith and Engle, 1927; Zondek and Ascheim, 1927). As reported by Smith and Engle, a marked sexual precocity may be induced in these animals by the foregoing method.

3. *Relation to thyroid.*—There is general agreement also that thyroid changes accompany hypophysectomy. It is not always clearly stated what is the histological nature of these changes. Crowe, Cushing, and Homans report thyroid hyperplasia. Dott reports acute hyperplasia after total ablation, followed by desquamation and appearance of colloid. Partial lesions he found to be followed by hypoplasia. (The foregoing for the dog.) Smith reports profound atrophy of the rat thyroid following hypophysectomy. There is general agreement that in the Amphibia absence of the anterior lobe of the hypophysis causes atrophy of the thyroid or, in the young larva, a failure of its development. Further, Smith and Smith (1922) succeeded in repairing the atrophic thyroid by parenteral administration of bovine anterior lobe. Uhlenhuth and Schwartzbach, (1927) regard the anterior lobe as in the nature of a control mechanism of thyroid activity in the salamander.

The strong sex-dimorphism of the birds, together with the need of further investigation in this group, make them profitable subjects for the study of pituitary function. The work of Goodale (1916), Zawadowsky (1922), Benoit (1923), and Domm (1924 and 1927) has helped to make clearer the nature of the action of the sex hormone in the domestic fowl, as well as the potentialities of the female gonad for differentiation toward either sex, as evidenced by its hormone production under experimental conditions. It should prove worth while to determine whether we have in the anterior pituitary hormone a stimulating mechanism for the gonads of birds also, and how an absence or deficiency of the hormone would affect the sex condition of the bird. The question of the general effects of hypophysis deficiency and its interrelations with the other endocrine glands is also worth investigation for the birds.

The writer is indebted to Professor Frank R. Lillie for the suggestion of this problem and for helpful suggestion and criticism during the course of the work. This opportunity is taken also to thank Dr. L. V. Domm and Dr. F. W. Appel for assistance in photographing birds, and Mr. Kenji Toda, the staff artist of the Department of Zoölogy, University of Chicago, for his assistance in preparing the photomicrographs appearing in this paper.

MATERIAL AND METHODS

1. *Material.*—The present investigation involves the removal or destruction of all or a part of the anterior lobe of the pituitary in the Leghorn breed of domestic fowl. The light-brown and dark-brown single-comb varieties have both been used, but most were light-browns. The advantages of this breed, as have been mentioned by Domm (1927), are its availability, medium size, strong sex-dimorphism, and comparative hardiness and vigor. The birds were, for the most part, obtained from a single source immediately after hatching and were reared in the laboratory. The age of the birds at the time of operation ranged from 3 weeks to 5 months, but most were operated in the period from 3 to 6 weeks. No operations on adult birds are reported here.

2. *Records and preservation.*—The weight and general condition at the time of operation were recorded for each bird, and a systematic check was made on all animals at intervals of a month thereafter, or less. Birds were also observed for any eccentricities of behavior. At death, a careful autopsy was resorted to, and material for future examination was fixed by the usual methods in Bouin's or formalin-Zenker's fluid. The gonads, adrenals, thyroids, parathyroids, and hypophyseal region were the tissues usually saved. For microscopic determination of extent of removal or injury to the hypophysis, the entire region was cut out as a cuboid block and fixed. The block included the sella turcica and overlying brain region. These blocks were later decalcified, imbedded, and serially sectioned through the sagittal plane. Both these and other tissues were stained with hematoxylin, with and without the eosin counterstain, and also with Mallory's triple stain.

3. *Operative procedure.*—Interpretations of experimental findings on the pituitary are reliable only in so far as the operative method has been controlled. The close approximation of this gland to the brain has made its uncomplicated removal difficult. Fichera used a method in his birds which differs from the bitemporal route of Paulesco (1907) and from the usual oral route. This consisted in making a ventromedian incision of the skin at the level of the angle of the jaws and in displacing the pharynx laterally, whereupon the hypophysis was approached through the floor of the sella turcica.

Fichera employed a cautery for this and relied upon destruction rather than removal.

The principal anatomical features of the bird pituitary have been described in the papers of Fichera (1905), Tilney (1911 and 1913), and several others; but for clarity it is well to present as graphically as possible the positions and relationships of the gland as found in the type of fowl used in this study. Figures 1 and 2

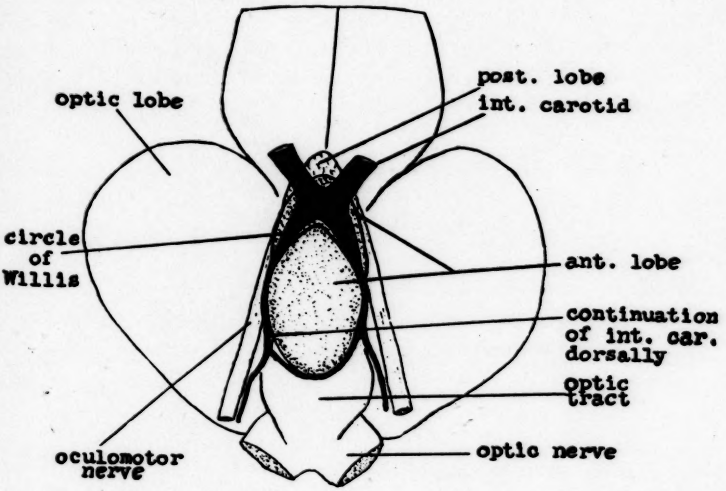


FIG. 1.—A sketch of a ventral dissection of the chick brain, to illustrate pituitary relationships.

illustrate in a diagrammatic way the points of importance. The form of the avian skull makes the ventral approach to the hypophysis the most practical one. Fichera developed his method for the purpose of avoiding the contact of oral and cranial cavities, thus lessening the likelihood of infection. In this study, however, the more direct approach through the opened mouth was chosen, in light of the comparatively great resistance of birds to infection. Both the method of Fichera and a possible approach through the orbit were also tried and abandoned as being less satisfactory. All usual precautions to preserve asepsis were observed—sterilization

of instruments, etc. Obviously, the operative field was not strictly sterile in the open mouth, but either macroscopic or microscopic signs of infection have been rare.

The operation was carried out under a Spencer dissecting binocular microscope, with the bird on its back and under ether anesthesia. A median incision was made in the mucous membrane from the tubal fissure to the posterior end of the palatine fissure. The wound was spread by forceps held in the left hand, and transverse

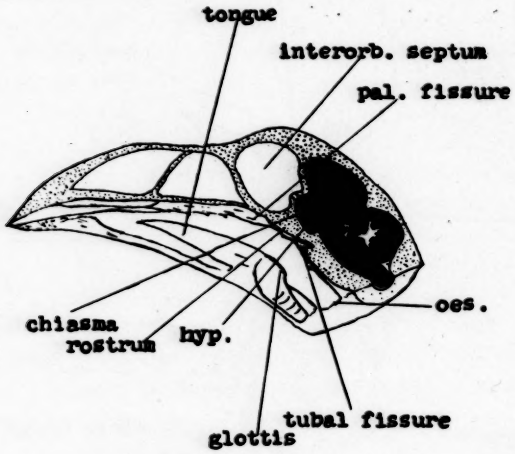


FIG. 2.—A sketch of a median sagittal section of the chick head, to illustrate the relationship of the pituitary to the mouth, skull, and brain. The operation route was through the rostrum.

cuts were then made in the rostrum by iridectomy scissors. The intervening cancellated bone was easily removed with fine forceps until the floor of the sella turcica was reached. This was then dissected off quite carefully with fine needles, as a slight misstep precipitated considerable bleeding. The path of the foregoing approach is approximately traversed by the label line to the hypophysis in the diagram of Figure 2. A longitudinal slit was next made in the membranes covering the gland, exposing its ventral surface. At this time the circulating blood in the gland parenchyma was easily observed under the microscope, giving an idea of the clearness of the

field. To the exposed surface was applied a small glass cannula, connected by rubber tubing to a filter pump, and the gland removed by suction. Some indication of the completeness of removal was gained by observation at the moment, as well as immediate examination of the site. Usually an electric cautery was applied, for the destruction of possible remaining fragments, though this was intentionally avoided in some cases as a check upon its effect. The removed tissue was recovered and fixed for sectioning, and in all except the earlier experiments the hypophyseal region of the bird was serially sectioned after death.

4. *Terminology and scope of experiments.*—These experiments have been confined to the removal of the "anterior lobe" only, and the subsequent record of the birds. There is lack of agreement as to what constitutes the anterior lobe of the bird hypophysis, however, hence a brief discussion seems desirable. Tilney (1913) describes the pituitary as consisting of *pars neuralis* and *pars buccalis*. The pars neuralis includes the eminentia saccularis, the infundibulum, and the infundibular processes; the pars buccalis consists of a *pars infundibularis*, closely investing the infundibular stalk, a *pars tuberalis*, partly covering the ventral surface of the eminentia, a *pars juxtaneuralis*, and a *pars distalis*. The pars juxtaneuralis apparently is the homologue in Tilney's description of the "pars intermedia" of mammalian pituitary. He describes a small lumen between this and the pars distalis of the fowl, which I am unable to confirm in the Brown Leghorn breed. The two portions are continuous, the whole forming a somewhat two-lobed mass, easily separable from the neural elements, the internal carotid arteries passing laterally in the grooves between the two "lobes" (see again Fig. 1). Figure 3 is a median sagittal section through the pituitary region of an adult bird, illustrating these points. Histologically the two regions present a somewhat different appearance, the portion nearest the neural part of the gland having a greater number of eosinophile cells, but the two intergrade with no sharp line of distinction. Therefore, in this paper the entire main mass of the epithelial portion is referred to as "anterior lobe," and this is the part removed. The posterior lobe is here referred to as including the infundibular stalk with its expanded extremity, which in the fowl

is lobulated and contains cavities in communication with the third ventricle through the stalk. Infundibularis and tuberalis portions, being impractical for removal, were left intact along with the posterior lobe.

Extirpations were performed upon young birds of both sexes. These were done in several series, a series consisting of a lot of birds hatched on the same day; they were thus approximately of the same age when operated. It is not deemed necessary to present the results in the form of series, but rather in the form of tables, summarizing all experiments for points of interest.

RESULTS

I. COMPLETE REMOVAL

a) *General symptoms.*—In all cases of complete removal of the anterior lobe, the bird died within 10 days. Immediately following the operation the birds usually recovered, perhaps eating and drinking by night of the same day operated. Many, however, remained quiet, with drooping wings and closed eyes. Fichera, who observed these same symptoms, probably correctly interprets them as due to the operative act. Nevertheless, cases of uncomplicated removal were often indistinguishable from normal birds after 24 hours. When they continued to be normal, or lived indefinitely, they subsequently proved to be cases of only partial removal. The others either died suddenly or showed droopiness and apathy for a while before death. The protocols of a few cases are presented below.

♂70. Age 44 days, weight 144 grams. Operated June 23, 1927. As far as observed, removal was complete, and gland was preserved for sectioning. Bird placed in pen as recovered on June 24. Died June 28, immediate symptoms of death not being observed. Autopsy: wound healed over, no sign of infection; no anterior lobe tissue macroscopically visible.

♂52. Age 51 days, weight 175 grams. Operated June 30, 1927. Removal appeared complete, though the gland was recovered in fragments. Returned to pen July 4 as recovered. Died July 7. Autopsy: wound healed; no infection; slight protrusion at hypophysis site—probably posterior lobe.

♂68. Age 45 days, weight 147 grams. Operated July 10, 1927. Anterior lobe removed intact and fixed in Bouin's. Bird bled considerably. Appeared recovered by July 12, and returned to pen. Died July 17. Autopsy: no signs of anterior lobe; no infection; and wound was healed.

♀48. Exact age unknown (secured as a pullet), but about 8 weeks; weight

317 grams. Operated October 2, 1927. Gland removed entire and preserved. No excessive bleeding. Died October 11, having been droopy several days previously. Autopsy: no observable disease; hypophysis site clean and normal in appearance.

♂17. Age 46 days, weight 152 grams. Operated July 8, 1927. Removal appeared complete, and gland preserved in Bouin's. Bird seemed normal July 8 and 9. Morning of July 10, bird was droopy, at intervals becoming excited and turning around in the cage (a phenomenon observed in unoperated birds occasionally, and at present of undetermined cause). Died in coma about noon. Autopsy: clean removal macroscopically.

The viscera of all these birds were examined for infections or other abnormalities which might have contributed to their death. Where nothing is reported, it may be assumed the animal was normal as far as could be determined.

b) Evidence of complete removal.—While macroscopic examination is not to be relied upon for *proof* of complete removal, it is felt that some evidence is lent to the cases of microscopically proved removals by the earlier cases of this study, in which the serial sections were not resorted to. A check in a series of thirty-two cases which were all examined microscopically showed that the protocol based on macroscopic examination at the time of operation agreed with the later microscopic findings in twenty cases, was in error in only four, and was doubtful in eight (i.e., the extent of removal uncertain at time operated). In Table I is given a summary of cases reported upon in this paper, with survival period correlated with degree of hypophysectomy. It will be noted that deaths in the first 2 days affect all cases about equally, and this, together with information in the protocols, makes it probable that the majority of these succumbed to operative injury. In the remainder of cases, it is apparent that deaths within the first few days fall preponderantly among those cases thought complete removals, while the indefinite survivors prove to be cases of partial removal.

c) Is death due to the operative act?—Table I makes it clear that birds with anterior lobe fragments have survived, whereas those lacking the gland entirely died within 2 weeks. Obviously, if death is due to the operative act, it must be more conducive to death when the gland is totally removed than when only a portion is removed. Experience in operating has shown that in general the partially removed gland has been the one to cause difficulties. If it does not

slip out at once in a single piece, greater efforts must be made to remove the fragments. This results in longer anesthesia, greater traumatization, greater hemorrhage, and more cauterization. These factors might therefore be expected to work against the survival of partial cases rather than in their favor. As all operations are subject to the same likelihood of infection, a difference of death-rate is not explainable on these grounds. In addition, operations were made as severe as possible without destruction of the gland in control birds without any cases of fatal results.

TABLE I

SURVIVAL PERIOD COMPARED TO DEGREE OF HYPOPHYSECTOMY*

SURVIVAL IN DAYS	DEGREE OF HYPOPHYSECTOMY			
	Partial		Complete	
	Macroscopically Observed	Proved Microscopically	Macroscopically Observed	Proved Microscopically
1	9	1	6	3
2	7	3	6	5
3	3	2	10	6
4	0	0	4	2
5	1	1	4	2
6	0	2	6	1
7	0	0	3	0
8	0	1	1	1
9	0	1	2	1
10	2	2	2	0
11–14	1	1	1	0
14+	10	23	2	0

* A summary of cases of hypophysectomy, comparing partials with completes as to survival period. "Macroscopically observed" means that the site of operation and the removed gland were examined to determine extent of removal, without recourse to microscopic sections. "Proved microscopically" means that the exact extent of removal was determined by sectioning the region at autopsy of bird.

With a view to determining as well as possible the extent of injury to the hypophyseal region by the operation, this region of thirty-five operated birds was dissected as a whole after death, decalcified, and serially sectioned. Only three of these birds had survived more than 10 days; one of these was an operated control, and the other two proved to be partial removals. Of the remaining thirty-two, six showed evidence of lesion. In four of these the injury appeared to involve only the posterior lobe. The writer has cases of partial removal with indefinite survival which have shown as severe

injury to the posterior lobe as these four cases. It is impossible to say whether any of this apparent injury has in reality been caused by the process of preparation. From the histological evidence, it is the writer's opinion that in the great majority of cases examined the ventral surface of the brain presents a normal intact appearance, including an intact third ventricle. The internal carotids can also be traced through the series in their passage to the brain.

It is recalled that Fichera (1905) had four cases of survival which he considered to have been completely devoid of anterior lobe, though he relied upon destruction rather than removal. In the light of this evidence, together with the survival of hypophysectomy obtained with other animals, it seems better to leave the question of indispensability of anterior lobe in the bird open for the present. Figures 4 and 5 are presented in partial substantiation of the foregoing discussion. They illustrate an intact hypophyseal region of a bird dying following hypophysectomy, and a removed gland (median section) to show its wholeness, though the action of the suction cannula usually tends to fragment the gland more than it was at the moment of its removal.

PARTIAL REMOVAL

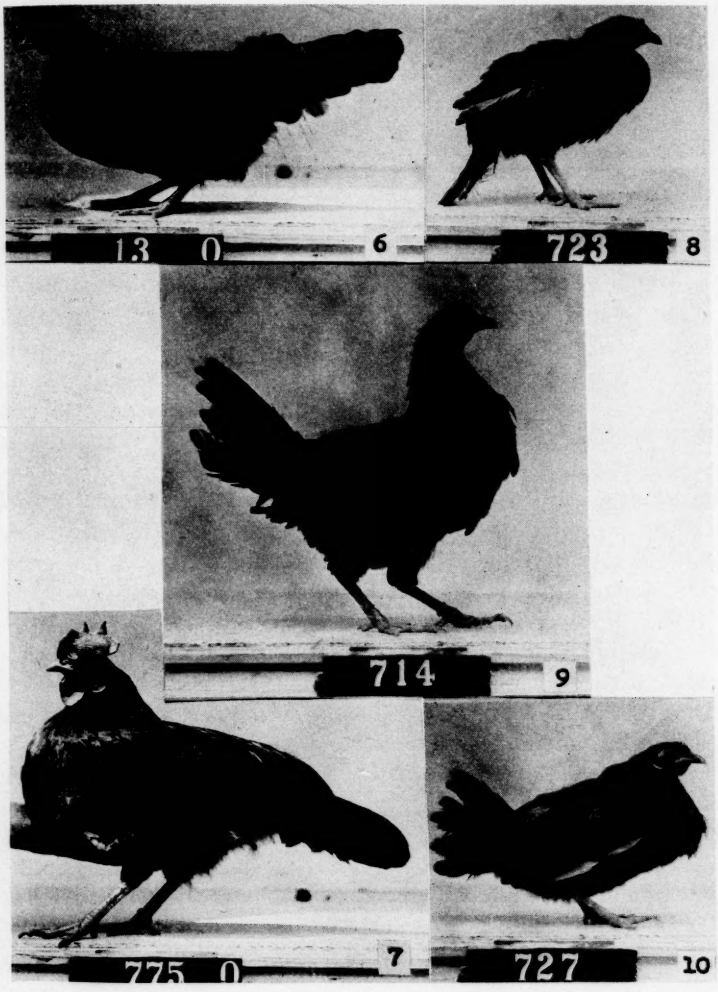
a) *General effects.*—As has been said, cases with some anterior lobe present may exhibit indefinite survival, i.e., live considerably over 2 weeks, or until sacrificed. Many of these cases have become indistinguishable from normal birds. They may be dismissed as not having deficiency of anterior lobe to the extent of producing effects. Others, however, have been easily distinguishable after a few weeks, by the following symptoms:

Recovery from the operation is complete, except that the birds are a little less active than normal, often standing quietly about the pen. In the course of a few weeks the weight of these birds is appreciably less than that of their controls, and they are of smaller proportions, more notably in the head and legs. Heads appear especially small, though this effect may be in part due to smaller head furnishings. The legs are of smaller diameter and shorter. Figures 6 and 7 show an operated bird and its control, illustrating these points. See also Figures 8 and 9, another pair, photographed when four months old. The control bird weighed 819 grams; while the other,

FIG. 3.—Photomicrograph of a median sagittal section of a dissection of the pituitary region of an adult female chick. Note large solid epithelial mass with its membranes, the hollow neural lobe, its cavity continuous with the third ventricle. ×25.

FIG. 4.—Photomicrograph of a median sagittal section of the pituitary region of ♀752. Shows intact posterior lobe, with connective tissue stroma on site of anterior lobe but no anterior lobe tissue present. Bird was 3.5 weeks old; 4 days after the operation. ×70.

FIG. 5.—Photomicrograph of a median sagittal section of the anterior lobe as removed from ♂765, 3 weeks old, to illustrate intactness of removal. ×70.



FIGS. 6–10.—Photographs, taken at equal distances, of operated birds and their controls. Figures 6 and 7 are an operated bird and its control, both over a year old, No. 13 having been hypophysectomized when about 85 days. Note small head furnishings, short legs, and general smaller size. Bird has not laid. Figures 8 and 10 are females operated at 2 weeks, while Figure 9 is their normal control; all photographed when 4 months of age.

operated 79 days previously, weighed 421 grams. Another operated bird of the same age, shown in Figure 10, weighed only 346 grams on the same date. There is very evident lack of preening, which is merely indicative of a bird in poor physiological condition. The same may be said of white feathering, which frequently occurs among these birds. The birds shown in Figures 8 and 10 illustrate these features.

Figures 11 and 12 show a normal control cock of 11½ months, and an operated male of identical age but hypophysectomized when 10 weeks old. The control weighed 1,606 grams when photographed, while the stunted animal weighed 936 grams. None of the excessive adiposity symptoms so often associated with hypophysectomy in mammals is noted in these birds; they are of about the normal adiposity of the pen, are not emaciated, but are smaller proportioned. In Table II is a summary of cases of retarded growth compared to normal controls. In addition, there were a number of other cases of definite retardation for which there were no comparable control data on weight. It is recognized, of course, that weight is a very variable quantity in birds; but in the absence of special disease or emaciation, it is indicative of the smaller size of the operated birds.

b) *Effects on the genital system and secondary sex characters.*—Originally it had been hoped to be able to control in some degree the plumage changes of the birds by an inhibition of the genital system as a result of hypophysectomy. Lacking survivals after complete loss of anterior lobe, the cases of stunting still offer material for the examination of genital system effects. It was found that the retarded birds retained the juvenile feathers for a much longer period than normal controls. The cock shown in Figure 12 still showed a scattering of juvenile feathers (not appearing to advantage in the photograph). The adult plumage should normally be acquired at 3–4 months of age. That this delay is not caused by any setback due to the operation is demonstrated by the operated controls, of which Figure 11 is an example, which acquire their adult plumage at the normal age. Even in animals with a portion of the anterior lobe removed, the plumage changes are normal if the remaining fragment of anterior lobe was large, these cases being indistinguishable from normal birds in size and plumage.

FIGS. 11–12.—Figures 11 and 12 show an operated control male and a male hypophysectomized at 76 days; both a year old when photographed. ♂8, the hypophysectomized bird, shows smaller head furnishings, smaller size, lighter weight, and retains some juvenile feathers (not apparent in the photograph); internally it has small but mature testes.

Internal examination of these cases of retardation, either at autopsy or by exploratory operation, has revealed in some instances a smaller ovary in the female, and in a few cases somewhat smaller testes in the male. The writer regards it as questionable whether these size differences in the gonads approach anything like the specific inhibition of the genital system found to follow the removal of

TABLE II

CASES OF STUNTING AS COMPARED TO CONTROLS*

Case No.	Age at Operation (Days)	Weight at Operation (Grams)	Age at Last Weight (Days)	Last Weight (Grams)	Comments
♂55	56	270	189	750	Runty; good condition
Control ♂29	56	222	174	1,107	Normal male
♂11	52	235	157	900	Somewhat smaller
Control ♂56		305	155	1,370	Normal male
♂8	76	287	353	936	Runty
Control ♂15		318	353	1,606	Normal male
♀13	85†	317	305	831	Small proportions
♀19	85†	231	310	1,071	Slight stunting
Control ♀37			287	1,346	Normal, laying female
♂729	58	308	112	387	Weak and stunted
Control ♂712			82	462	Younger bird
♀727	47	165	127	346	Runty; white feathers
Control ♀714			127	819	Normal female
♀711	46	172	94	260	Runty
♀705	44	186	94	218	Runty
Control ♀719			94	387	Normal female
♀723	47	168	270	711	Runty; compare Control ♀37

* A table of weights of controls and stunted birds. The weight is a reliable index of retardation only when taken in consideration with the general condition of the bird, of course.

† Approximate age; exact age unknown; received as a pullet.

the anterior lobe in mammals (cf. Crowe, Cushing, and Homans [1910]; Smith [1927]; etc.). The retarded ovary shows small follicles, but no large ones. The ovary of the operated bird shown in Figure 6, for example, showed no follicles more than 2 mm. in diameter, though the bird was over a year old, and should have been laying, as was her control. It should be noted, however, that follicles of 2

mm. are not found in the typical "juvenile" ovary, and this bird may eventually ovulate, as others have done if surviving long enough. If this hen does ovulate, all cases will have proven to be retardations rather than permanent inhibitions.

In the male, microscopic examination of the testis has revealed a normal spermatogenic cycle, resulting in the production of spermatozoa, in spite of the smaller size of the gland. The testes of the cock shown in Figure 12 measure approximately 20×6 mm., whereas a normal cock of like age should have testes of about 45×20 mm. (length by diameter). Nevertheless, these small testes exhibit spermatozoa.

Head furnishings of retarded birds are also smaller. Combs of females are normally enlarged and red during ovulation periods. In retarded birds possessing only small follicles the comb correspondingly lags behind that of the control. Examination of the head furnishings of the birds shown in Figures 6 and 7 will verify this point. Typical measurements of the comb in retarded males as compared to controls have also shown smaller combs. Thus the comb of ♂55 measured 19×7 mm. (greatest length by greatest height), while its control was 51×25 mm; but again this size difference appears as a lagging rather than a specific inhibition. The head furnishings are subject to variations in the general condition of the bird, and there is also some evidence that the size of the comb may be at least in part conditioned by the size of the body. In short, it seems more probable that the smaller comb of stunted birds is only a reflection of their general condition.

c) Effects on other endocrine organs.—

1. The Thyroid: One of the most constant features of the partially hypophysectomized birds has been the small size of their thyroids, particularly in the markedly stunted birds. Though normally a variable organ as regards weight, reference to Table III shows that the thyroids of experimental birds fall largely below the range of variability of the controls. The gland in some cases has been reduced to a very flat, pale, and inconspicuous strip of tissue lying on the jugular vein.

Examination of the histological picture of these has shown a marked tendency to the accumulation of colloid, with a correspond-

ing decrease in the relative amount of epithelium. In some of the more distended vesicles the epithelium is reduced to a very thin

TABLE III

THYROID WEIGHTS IN EXPERIMENTAL AND CONTROL BIRDS*

No.	Age in Days	Thyroid Weight in Grams	Month of Death
	2 Lobes		
♂55	189	Very small	Oct.
♂78	164	0.0406	Nov.
♂88	163	0.0506	Oct.
♂16	163	0.0360	Oct.
♂42	162	0.0538	Nov.
♂93	162	0.0490	Nov.
♂14	161	0.0410	Oct.
♂11	158	0.0356	Oct.
Control ♂56	155	0.0922	Oct.
Control ♂29	174	0.0622	Oct.
	1 Lobe†		
♂84	285	0.0198	Apr.
♂2	130	0.0102	Oct.
♂729	112	0.0136	Jan.
♂703	147	Very small	Dec.
Control ♂44	278	0.3620	Mar.
Control ♂712	171	0.0954	Apr.
Control ♂713	171	0.0407	Apr.
Control ♂716	171	0.0566	Apr.
♀19	310	0.0470	Apr.
♀85	288	0.0340	Apr.
♀7	242	0.0248	Apr.
♀33	217	0.0106	Jan.
♀727	146	0.0098	Mar.
♀711	93	0.0194	Jan.
♀705	90	0.0166	Jan.
Control ♀37	287	0.0932	Apr.
Control ♀64	287	0.0922	Apr.
Control ♀701	282	0.0680	Apr.
Control ♀715	98	0.0534	Jan.

Max. (1 lobe) operated ♂ = 0.0198 gm.; Min. = 0.0102 gm.
Max. (1 lobe) control ♂ = 0.3620 gm.; Min. = 0.0311 gm.
Max. (1 lobe) operated ♀ = 0.0470 gm.; Min. = 0.0106 gm.
Max. (1 lobe) control ♀ = 0.0932 gm.; Min. = 0.0680 gm.

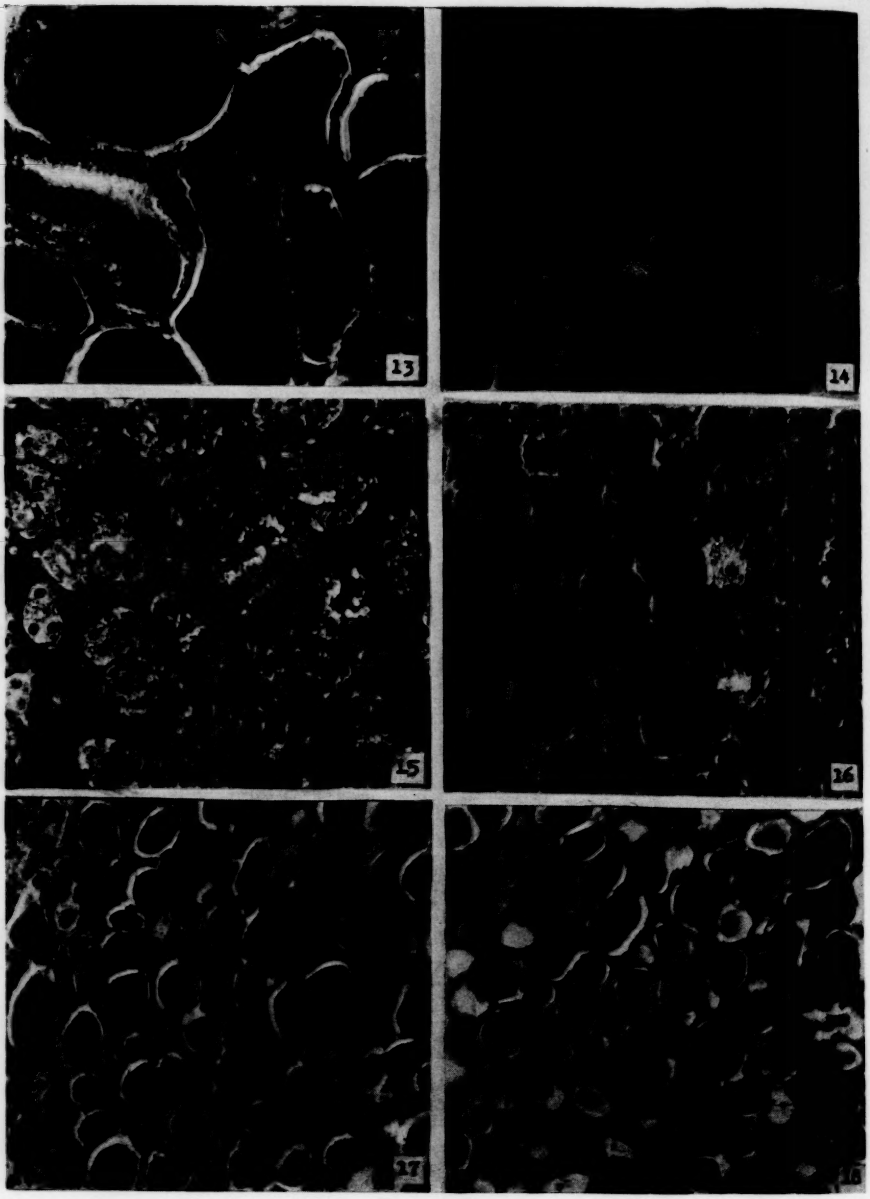
* Comparison of thyroid weights in experimental and control birds. One set indicates the combined weight of both lobes. The other set gives the weight of one lobe only as the other lobe had been previously used for microscopic study.

† Only one lobe weighed, in 70 per cent alcohol; other not available.

layer of cells. In sectioning, the paraffin ribbon of these glands has a distinctly "grainy" appearance, from the hardening of the colloid in these vesicles. Figures 13 and 14 are photomicrographs of thyroid sections of ♂ 14 and ♂ 55, while Figure 15 shows the thyroid of a normal control. Figure 16 is the thyroid of a normal 21-day-old control female, while Figures 17 and 18 show the thyroids of an operated female and male of the same lot shortly after the operation.

Table IV summarizes the thyroid situation, giving average thickness of epithelium and average ratio of this to lumen of the vesicle, expressed in arbitrary micrometer units. The ratio expresses a better idea of the relative amount of glandular tissue present in proportion to colloid, especially as it is remembered that the absolute weights are less (cf. Table III). There was no close relation found to exist between this accumulation of colloid and the size of the remaining anterior lobe fragment, except that in general the birds exhibiting the greatest stunting proved to have the least hypophysis and the most abnormal thyroids. In Table V is summarized a series of thirty-one cases, correlating their survival period with thyroid condition. This presents some evidence as to when the changes begin in the thyroid. There is a slight indication of the accumulation of colloid within 48 hours after the operation, but this undoubtedly overlaps the normal variation in colloid content of the glands. By 3 days a distinct increase of colloid can be seen. In general, a direct ratio is found between time since operation and accumulation of colloid. It is recognized that the figures of the tables are only an attempt to present in something approaching a quantitative fashion the histological changes best observed in the slides.

The question may be justly raised as to whether the foregoing described changes of the thyroid are due to deficiency of anterior lobe secretion or to some other factor. The thyroid is quite variable in size in normal birds, and in sick and emaciated birds small thyroids are frequently observed. Dr. Mary Juhn, of the Whitman Laboratory, has found normal female thyroids to possess considerable colloid at some seasons (unpublished data). In view of these facts, it seems unwarranted to assume a specific relationship between the thyroid and hypophysis. It may be more probable that



FIGS. 13–18.—Photomicrographs of thyroids of hypophysectomized birds and controls. Original ×400, reduced one-third. Figures 13 and 14 are from operated birds ♂14 and ♂55, respectively, while Figure 15 is their normal control, ♂44. Figure 16 is the thyroid of a normal 3-week-old female, while Figures 17 and 18 are of ♀752 and ♂739, hypophysectomized at 3 weeks, and thyroids fixed 4 and 6 days later, respectively.

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the changes in the general metabolic condition of the birds are reflected by thyroid atrophy.

TABLE IV

ACCUMULATION OF COLLOID IN THYROID OF HYPOPHYSECTOMIZED BIRDS*

Case No.	Age in Days	Thyroid Epithelium	Ratio of Colloid to Epithelium	Month of Death
♂84	285	2.30	5.2	Apr.
♂55	189	0.72	15.5	Oct.
♂88	163	0.90	25.6	Oct.
♂16	163	1.00	15.8	Oct.
♂42	162	1.10	12.1	Nov.
♂93	162	1.00	12.3	Nov.
♂14	161	0.95	16.3	Oct.
♂11	158	1.20	8.9	Oct.
♂703	147†	0.90	7.6	Dec.
♂2	130	0.90	9.7	Oct.
♂729	112	1.00	8.1	Jan.
Control ♂44	278	4.80	.32	Mar.
Control ♂29	174	2.70	3.63	Oct.
Control ♂712	171	5.10	1.62	Apr.
Control ♂713	171	2.75	4.80	Apr.
Control ♂716	171	4.10	1.55	Apr.
Control ♂56	155	2.30	6.70	Oct.
♀19	310	3.30	3.00	Apr.
♀32	288	4.10	1.36	Apr.
♀85	288	5.20	1.04	Apr.
♀7	242	3.00	1.66	Apr.
♀727	146	1.00	9.70	Mar.
♀711	93	3.00	1.00	Jan.
♀705	90	0.50	23.40	Jan.
Control ♀37	287	3.60	0.48	Apr.
Control ♀64	287	4.80	0.56	Apr.
Control ♀701	282†	5.30	1.88	Apr.
Control ♀776	42	2.90	1.00	Nov.
Control ♀589	112	5.50	1.29	Jan.

Max. ratio colloid to epithelium, operated ♂ = 25.6; Min. = 5.2
Max. ratio colloid to epithelium, control ♂ = 6.7; Min. = 0.32
Max. ratio colloid to epithelium, operated ♀ = 23.4; Min. = 1.00
Max. ratio colloid to epithelium, control ♀ = 1.88; Min. = 0.48

* A study of the accumulation of colloid in the thyroid of hypophysectomized birds. The thyroid epithelium is here expressed as its height or thickness in arbitrary micrometer units. Ratio of colloid to epithelium is a better index of colloid accumulation, the diameter of the colloid mass being taken in the same arbitrary units. Figures are an average of 40 readings of cross-sections of vesicles.

† Approximate age; exact age unknown; secured as young bird.

2. Parathyroids and Adrenals: These two glands have been preserved and examined along with the thyroids and gonads, though less extensively than the latter two. There were no macroscopic

differences to be noted between the glands of experimental and control birds. Occasionally the parathyroids have appeared to be lighter, and again they have seemed to be somewhat enlarged; it

TABLE V

THYROID CHANGES COMPARED TO TIME SINCE HYPOPHYSECTOMY*

Case No.	Survival in Days	Thyroid Epithelium	Ratio of Colloid to Lumen	Anterior Lobe
Control ♂		2.28	2.10	Normal
Control ♂		2.40	2.40	Normal
Control ♀		2.55	1.89	Normal
Control ♀		3.00	1.46	Normal
♂737	0.5	1.80	3.20	None
♂746	1.0	1.80	4.00	None
♂747	1.0	2.00	2.60	None
♀735	1.5	1.80	3.20	None
♂745	1.5	1.80	3.30	None
♂748	1.5	1.70	3.60	None
♀750	1.5	2.00	2.50	None
♀753	1.5	1.80	2.20	Fragments
♀767	1.5	2.20	2.20	None
♂740	2.0	1.80	2.50	None
♂763	2.0	0.80	6.70	Small mass
♂738	2.5	1.50	4.60	None
♀744	2.5	1.80	3.20	Small mass
♂749	2.5	1.50	4.10	None
♀754	3.0	1.10	6.00	Few cells
♂756	3.0	1.00	7.00	None
♂758	3.0	1.00	7.30	None
♂761	3.0	1.80	3.30	None
♂766	3.0	1.00	7.00	None
♀768	3.0	1.90	3.00	None
♂734	3.5	1.30	6.70	None
♀752	4.0	0.70	9.80	None
♂736	4.5	1.00	7.40	Few cells
♂743	5.0	0.90	8.30	None
♂762	5.0	0.80	8.50	None
♂739	6.0	0.50	16.00	Small mass
♂741	6.0	1.00	7.20	Small mass
♀764	6.0	1.10	5.10	None
♂765	8.0	0.80	6.70	None
♀755	8.5	2.00	1.90	Small mass
♂751	9.0	0.80	9.00	None

* Units the same as for Table IV. All birds were 20–30 days old when operated. Figures calculated as before, from 10 readings (these being fairly uniform).

therefore appears that these are only environmentally-caused size differences. Histologically, no differences from the normal have been detected in either parathyroid or adrenal of the cases examined.

DISCUSSION

As mentioned in the introduction, there is no general agreement as to the indispensability of the hypophysis. The more modern trend has been to find its removal not incompatible with life. There remains, however, the possibility of differences of capacity in this respect in different classes of animals. It is perhaps, after all, a minor point, as the main interest lies in determining what are the positive effects of the gland's secretion in the living animal. The writer's results have not confirmed those of Fichera, who destroyed the gland by means of a cautery. In view of the extreme difficulty of checking every other possible cause of death, one does not feel justified in stating that it is impossible for brown leghorns to survive the operation, though no other cause of fatality has been apparent.

The question may also be raised as to how much anterior lobe is sufficient to maintain the life of these birds. The evidence on this point has been complicated by the problem of hypertrophy of remaining fragments. Removed glands may appear fairly intact at sectioning, and yet considerable masses of anterior lobe tissue be found at autopsy. This is indirect evidence of hypertrophy, but the point has not been specifically investigated. The pars tuberalis also complicates the situation; and as Tilney (1913) points out, it seems unlikely that this portion of the gland can ever be completely removed without serious injury to the brain. Does this portion hypertrophy in some forms; and if so, would its secretion compensate for the deficient anterior lobe? Koster (1927) concludes from a study of serial sections of the hypophysis region of hypophysectomized dogs that the pars tuberalis has hypertrophied. The writer has been unable to find any evidence of hypertrophy of the tuberalis or infundibularis portions of the gland in the fowl. Nevertheless, in order to produce any symptoms of anterior lobe deficiency, it has been necessary that there be only a very small portion of the original gland present.

The retardation of development indicated in Fichera's report does not appear to have been great. His four cases of complete destruction averaged 650 grams after 4 months, as compared to a normal average of 720 grams; age is not stated. The results here reported have shown a greater degree of retardation than this, and

it appears that in birds as elsewhere, deficiency in anterior-lobe secretion results in an interruption of the normal growth processes of the young animal.

Fichera reports negative findings as to the effects of his operation on other endocrine organs. He particularly noted the gonads. The results of these experiments agree in failing to find any specific inhibiting effect of lack of anterior lobe, though there is a noticeable retardation evidenced by the smaller size of the gonad in the stunted animals. It may be possible that anterior lobe deficiency has produced in these cases marked growth differences, but the hormone is still present in sufficient quantity to maintain the genital system. Unless birds can be made to survive *complete* loss of the gland, the question of the specific inhibition of the gonads cannot be definitely settled. If Fichera's four cases be relied upon, however, this author finds no changes in the gonads. This is in contrast to the infantile condition produced in dogs by Crowe, Cushing, and Homans, by Bell, and by Dott; and in rats by Smith.

The converse experiment, the injection of anterior lobe substance, as practiced by Smith and Engle (1927) and Zondek and Ascheim (1927), has served further to substantiate the conception of a gonad-stimulating function of the hypophysis in mammals. Under these conditions, the gonad (ovary) is stimulated to produce a larger number of eggs, and the genital tract is matured more rapidly in the female; similar changes of a lesser degree occur in the male. Preliminary experiments by the writer have indicated that the fowl is not so immediately and profoundly responsive to hypophyseal transplants as is the mammal; this is to be checked by further experiment.

The results of these experiments confirm Fichera in indicating no effect of hypophysectomy on other endocrine organs of the bird with one exception—the thyroid. Smith (1916) reports a regression in size of the adrenals and parathyroids of the rat, but there is no evidence of this in these experiments on the bird. For the thyroid there is general agreement of disturbed function in Amphibia and mammals. This is generally described as being of the nature of atrophic changes. Thus Smith (1916), Smith and Smith (1922), and others for the frog; Ascoli and Legnani (1912), Bell (1917), Houssay

and Hug (1921), Dott (1923), and Smith (1927) for the mammal. Both Dott and Smith report these changes as being a flattening of the epithelium and decrease in size. Ascoli and Legnani report the accumulation of colloid. The smaller size of the thyroid in birds also agrees with the work on Amphibia, where the gland is seriously inhibited by removal of the anterior lobe primordium in the larva (cf. Smith, 1916).

This investigation, then, has confirmed the findings of a majority of workers regarding the retarding effects on growth of anterior lobe deficiency. As to entire absence of anterior lobe, it has been impossible to secure survival of the birds indefinitely. The work has shown that while sexual maturity may be retarded by anterior-lobe deficiency, this does not appear to be specific in the bird, but seems more likely to be associated with a lowered metabolism, of which the stunted growth and atrophic thyroids are also an expression. A number of further possibilities present themselves for investigation before the final word can be said regarding hypophysis function in the bird.

SUMMARY

1. This investigation involves the study of 192 brown leghorn domestic fowl, of which 161 were hypophysectomized (anterior lobe only) by the oral route. There were 10 operated and 21 normal controls.

2. Birds did not survive more than 2 weeks following total removal. Serial section of the hypophyseal region of 52 of these cases did not reveal injuries or other causes to account for these deaths. They were not preceded by specific symptoms.

3. Partial hypophysectomy was not necessarily fatal, and if nearly complete, distinct retardation, with changes in the thyroids, resulted. Stunting occurred in 15 out of 35 cases where the survival period was sufficiently long to exhibit it.

4. Stunting was accompanied by delay in assumption of adult plumage and attainment of sexual maturity, though no specific inhibition of the gonads was produced.

5. Thyroid changes are: a decrease in size of the gland; an accumulation of colloid in the vesicles; and an accompanying flatten-

ing of the secretory epithelium. Evidence points to these changes being in progress by the third day following the operation.

6. There was no evidence of changes in parathyroids or adrenals.

7. No symptoms of deficiency were noted unless the remaining fragment of anterior lobe was very small in relation to the whole, but the fragment nevertheless appeared to maintain life.

LITERATURE CITED

ADLER, L. 1914. Metamorphosenstudien an Batrachierlarven. I. Extirpation endocriner Drüsen. A. Extirpation der Hypophyse. Arch. f. Entw. d. Org., XXXIX, 21–45.

ALLEN, B. M. 1918. The results of thyroid removal in the larvae of *Rana pipiens*. Jour. Exp. Zoöl., XXIV, 499–517.

ASCHNER, B. 1912. Über die Funktion der Hypophyse. Arch. f. die ges. Physiol., CXLVI, 1–146.

ASCOLI, G., and LEGNANI, T. 1912. Die Folgen der Extirpation der Hypophyse. München. med. Wochenschr., LIX, 518–21.

BELL, W. B. 1917. Experimental operations on the pituitary. Quar. Jour. Exp. Physiol., XI, 77–126.

BENOIT, J. 1923. Transformation expérimentale du sexe par ovariotomie précoce chez la poule domestique. C. R. de l'Acad. Sci., CLXXVII, 1074.

BROWN, C. G. 1922. The effects of complete extirpation of the hypophysis in the dog (preliminary report). Proc. Soc. Exp. Biol. Med., XX, 275–76.

CROWE, S. J.; CUSHING, H.; and HOMANS, J. 1910. Experimental hypophysectomy. Bull. Johns Hopkins Hosp., XXI, 127–69.

DANDY, W. E., and REICHERT, F. L. 1925. Studies on experimental hypophysectomy. I. Effect on maintenance of life. *Ibid.*, XXXVII, 1–13.

DOMM, L. V. 1924. Sex-reversal following ovariotomy in the fowl. Proc. Soc. Exp. Biol. Med., XXII, 28–35.

———. 1927. New experiments on ovariotomy and the problem of sex inversion in the fowl. Jour. Exp. Zoöl., XLVIII, 31–173.

DOTT, N. M. 1923. An investigation into the functions of the pituitary and thyroid glands. Quar. Jour. Exp. Physiol., XIII, 241–82.

EVANS, H. M. 1924. The function of the anterior hypophysis (Harvey lecture). Philadelphia: Lippincott.

EVANS, H. M., and LONG, J. A. 1921. The effect of the anterior lobe administered intraperitoneally upon growth, maturity, and oestrous cycles of the rat. Anat. Rec., XXI, 62–63.

EVANS, H. M., and SIMPSON, M. E. 1926. Effects of anterior hypophyseal extracts on the male. *Ibid.*, XXXII, 206.

FICHERA, G. 1905. Sulla distruzione dell' epofisi. Arch. di Biol. norm. e pat., LIX, 739–96.

GOODALE, H. D. 1916. Gonadectomy in relation to the secondary sex characters of some domestic birds. Carnegie Inst. Wash. Pub. No. 243.

HOGBEN, L. T. 1922. The effect of pituitary (anterior lobe) injections upon normal and thyroidectomized axolotls. Proc. Roy. Soc. Lon., XCIV, 204–15.

HOUSSAY, B. A., and HUG, E. 1921. Influencia de la hipofisia sobre el crecimiento. Rev. assoc. méd. Argent., XXXIV, 269. (Original not seen.)

KOSTER, S. 1927. Experimentelle Beiträge zur Kenntnis der Hypophysenfunktion. Nedersch. tijdschr. v. geneesk., LXXI, 2612. (Reviewed in Endocrinology, Vol. XII [1928].)

PAULESCO, N. C. 1907. Recherches sur la physiologie de l'hypophyse du cerveau. L'hypophysectomie et ses effets. Jour. de Physiol., IX, 441–56.

SMITH, P. E. 1916. Effect of hypophysectomy in early embryo upon growth and development of the frog. Anat. Rec., XI, 57–64.

———. 1926. Ablation and transplantation of the hypophysis of the rat. *Ibid.*, XXXII, 221.

———. 1927. The disabilities caused by hypophysectomy and their repair. Jour. Amer. Med. Asso., LXXXVIII, 158–61.

SMITH, P. E., and ENGLE, E. T. 1927. Experimental evidence regarding the rôle of the anterior pituitary in the development and regulation of the genital system. Amer. Jour. Anat., XL, 159–217.

SMITH, P. E., and SMITH, I. P. 1922. The repair and activation of the thyroid in the tadpole after hypophysectomy. Jour. Med. Res., XLIII, 267–84.

TILNEY, F. 1911. Contribution to the study of the hypophysis cerebri with special reference to its comparative histology. Mem. of the Wistar Inst. No. 2.

———. 1913. An analysis of the juxta-neural portion of the hypophysis cerebri, with an embryological and histological account of a hitherto undescribed part of the organ. Internat. Monatschr. f. Anat. u. Physiol., XXX, 258–93.

UHLENHUTH, E. 1920. Experimental giantism produced by feeding pituitary gland. Proc. Soc. Exp. Biol. Med., XVIII, 11–14.

UHLENHUTH, E., and SCHWARTZBACH, S. 1927. The morphology and physiology of the salamander thyroid. II. The anterior lobe of the hypophysis as a control mechanism of the function of the thyroid gland. Brit. Jour. Exp. Biol., V, 1–5.

ZAWADOWSKI, M. 1922. Das Geschlecht und die Entwickelung der Geschlechtsmerkmale. Moscow.

ZONDEK, B., and ASCHEIM, S. 1927. Das Hormone des Hypophysenvorderlappens. Klin. Wochenschr., VI, 248–52.